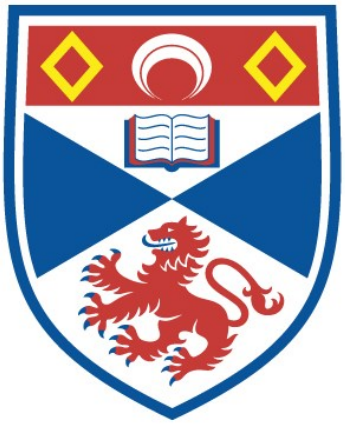
THE PROPERTIES AND DEVELOPMENT OF AXOPODIAL MICROTUBULES IN THE HELIOZOAN ACTINOPHRYS SOL

Colin Douglas Ockleford

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1973

THE PROPERTIES AND DEVELOPMENT OF AXOPODIAL MICROTUBULES IN THE HELIOZOAN *ACTINOPHRYS SOL*

by

Colin Douglas Ockleford

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submitted for the degree of
Doctor of Philosophy

August 1973

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University of St Andrews

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University Career

In October 1966 I started a four-year degree course at St Andrews University. This led to my graduation with an upper second-class Honours BSc in Zoology. I remained at St Andrews and took up a Science Research Council Studentship in the Zoology Department. In 1972 I spent a short time working in the Zoology Department, Edinburgh University. Since October 1970 I have researched into the axopodial microtubules of the heliozoan Actinophrys sol. The results of my research are presented here for the degree of Doctor of Philosophy.

Declaration

I declare that this thesis is the result of my own work. Where observations and experiments, performed by others, are referred to in the text they have been acknowledged. None of the material in this dissertation has been submitted by me for any other degree.

August 31st, 1973

..... (candidate) .....

Certificate

I certify that Mr Colin Douglas Ockleford has spent twelve terms at research work on the axopodial microtubules of the heliozoan Actinophrys sol, that he has fulfilled the conditions of Ordinance No. 16 (St Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

August 31st, 1973

(supervisor)

SUMMARY

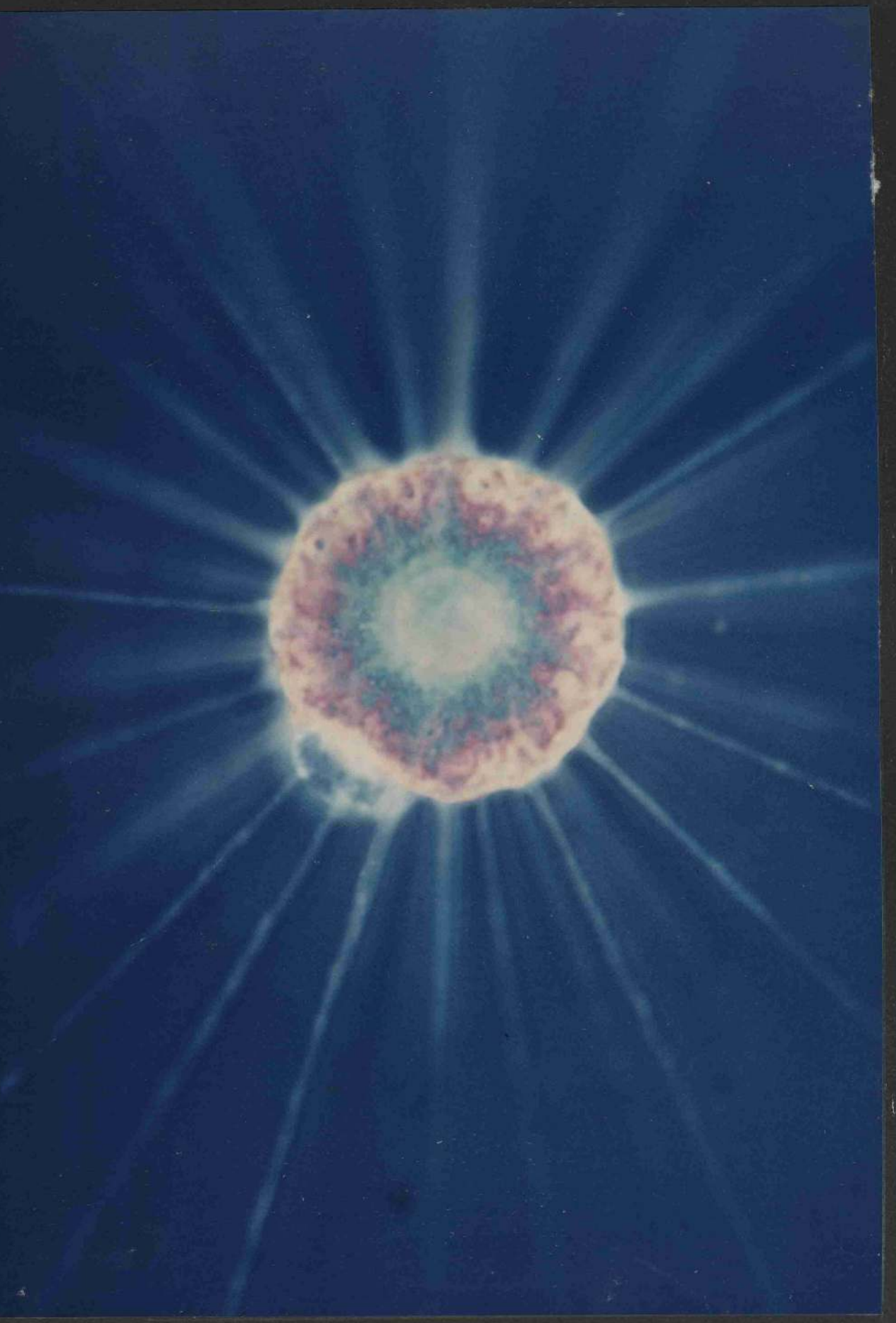
Long lengths of microtubules apparently break down very rapidly (100 μm in under a second) during an hitherto undescribed feeding response. Microtubular axopodia elastically resist mild bending along their longitudinal axes. They yield non-elastically when more severe bending is applied; bends are formed at certain points along their longitudinal axes. A polarised repair process returns bent axopodia to their normal straight form. Bends under repair move out along axopodia towards their tips. Employing these bends as markers it has been shown that breakdown of microtubules takes place at the tips of tubules at the distal ends of axopodia when axopodial shortening is induced with colchicine.

Evidence has been gained which suggests that microtubule growth and breakdown may be restricted to precisely localised areas within an organism. Apart from this crude control over the presence or absence of tubules in a certain position, it appears that the cell has provision for the re-deployment of prefabricated groups of microtubules.

A study of tubule regrowth and of the formation of patterned aggregates of microtubules after experimental disassembly suggests that tubule initiation, orientation and pattern formation occur as temporally separate processes. Pattern formation does not appear to depend on a pre-existing pattern of nucleating sites based on a two-dimensional template.

"In many cases it is the arrangement of the axial rods - the 'polar symmetry' of the entire organism - which lies at the root of the matter; and which, if only we could account for it, would make it comparatively easy to explain the superficial configuration."

D'Arcy Wentworth Thompson,
On Growth and Form (1942)



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GENERAL INTRODUCTION

The statement that microtubules are of fundamental biological significance would probably be considered unexceptionable by the majority of cell biologists. Any structures of integral importance to cell division, cell motility, intracellular transport, and the development of cell shape are surely worthy of the closest consideration.

Work on microtubular systems is progressing on several fronts. Biochemical characterisation, the description of the development of tubules, and the elucidation of microtubular function are three main "focal points" of current investigations. This thesis is the record of a detailed study of one microtubular organelle. The study has yielded new information which contributes to our understanding of microtubule development, structure, and function.

The organelle concerned is the axopodial axoneme of the heliozoan Actinophrys sol. An axoneme is the microtubular, central portion of an axopodium, which is a long thin cell extension. Actinophrys like the other members of the family Actinophyridii has no inorganic or chitinous surface coating.

From many points of view this organism is well suited to a study of this type. It is a free living protozoan which is easily cultured in the laboratory. It is radially symmetrical and has more than 100 axopodia protruding from its surface. Thus it is suitable for a detailed electron microscopic examination of axonemal organisation. There are few, if any, cases where part of a cell contains such a high proportion of microtubules which are so easily subjected to experimental analysis.

It has been possible to go some way towards answering the following

questions:-

1. Do long lengths of microtubule break down instantaneously under natural conditions?

2. What is the mechanism of the destructive action of colchicine on microtubules?

3. Are the initiation of microtubule assembly, determination of the pattern of tubule packing, and specification of tubule orientation controlled by distinct and separate mechanisms?

4. Are microtubules stiff enough to undertake cytoskeletal roles which have often been suggested for them?

5. Can current theories of tubule length determination account for all data reported here for heliozoan microtubules?

6. Are axopodial microtubules involved in heliozoan binary fission?

MATERIALS AND METHODS

(a) Isolation of Actinophrys sol (Ehrenberg, 1838)

Actinophrys sol (frontispiece) was obtained from Cameron Reservoir near St. Andrews. This organism is being maintained in culture at the Cambridge Culture Centre of Algae and Protozoa, Cambridge, England (List no. LB502/1). I thank Dr. F.C. Page for confirming my identification which is based on Kainer's (1968) key for heliozoans. The cultures of Actinophrys originate from a population of several founder organisms (Wichtermann, 1953).

(b) Culturing of Actinophrys

Actinophrys has been maintained in the laboratory for 2½ years by serial subculturing. The culture medium is made by boiling 100 ml of filtered Cameron Reservoir water with 3 wheat grains for 5 min. Boiling and filtering was undertaken to kill other organisms in the water collected from the reservoir. Fresh cultures were prepared by collecting at least 20 Actinophrys from a 2-week-old culture with a finely drawn glass Pasteur Capillary Pipette (Harshaw Chemicals Ltd.). The Actinophrys were then pipetted into a 9 cm diameter disposable Petri dish containing approximately 8 ml of culture medium and a boiled wheat grain. After inoculation a rich suspension of the ciliate Tetrahymena was added to the culture as food. To obtain "clean" cultures for electron microscopy the wheat grain was omitted. However in the normal course of culturing it provided nutrition for a population of small flagellates (Ochromonas) which were carried over in the pipette from the previous culture. These flagellates were preyed on by Actinophrys which multiplied more rapidly on this mixed diet than on Tetrahymena alone.

(c) Food organisms

Tetrahymena pyriformis (C.C.C.A.P. List no. L163/1W) was cultured axenically at 10°C in an aqueous solution of 1% proteose peptone and 0.1% yeast extract (Mackinnon and Hawes, 1961). Before addition to the Actinophrys cultures the ciliates were concentrated by mild centrifugation and resuspended in boiled and filtered reservoir water.

Several different food organisms were used to examine the method of prey capture in Actinophrys. These were from axenically grown stocks obtained from Cambridge and were not maintained in the laboratory by the author. They were concentrated by mild centrifugation, resuspended in boiled and filtered Actinophrys culture medium and used immediately after receipt.

TABLE I: Food organisms

	Source	List no.
Ochromonas danica	Culture Centre of Algae and Protozoa	L933/2
Ochromonas malhamensis	"	L933/1a
Ochromonas sociabilis	"	L933/3
Chlamydomonas reinhardii	Supplied by Dr. A.M. Sincock, Zoology Department, St. Andrews University	

(d) Light microscopy

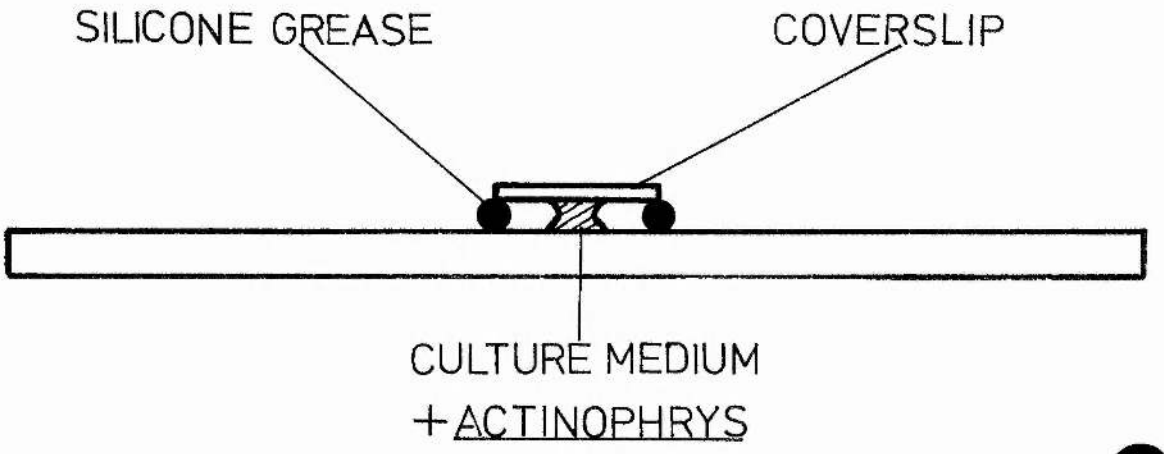
For observations on living Actinophrys a ring preparation was devised (Fig. 1). In such preparations organisms survived for 48 hr or more. A ring of silicone grease (Edwards High Vacuum Ltd.) was prepared by expulsion from an hypodermic syringe onto a glass slide. The needle of the syringe was shortened to a stump with a pair of wire-cutters in order to reduce the pressure required to eject the viscous grease through the long narrow passage. A small drop of culture medium containing Actinophrys was placed on the slide in the middle of the ring of silicone grease. A square No. 1 coverslip of side 2 cm was pressed down evenly onto the ring until the drop of culture medium met the coverslip

FIGURE 1

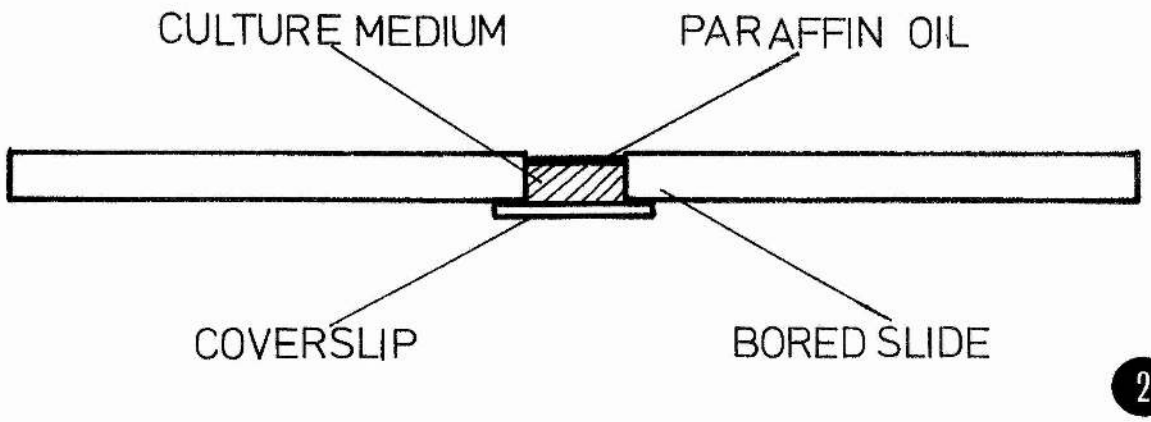
A "ring preparation": the type of preparation which was usually employed to examine living Actinophrys.

FIGURE 2

Illustrates the type of preparation most suitable for micromanipulation studies. The paraffin oil prevents evaporation of the culture medium.


SILICONE GREASE
COVERSLIP
CULTURE MEDIUM
+ACTINOPHRYS

1


CULTURE MEDIUM
PARAFFIN OIL
COVERSLIP
BORED SLIDE

2

to form a column. Vaseline, which is often used for sealing cytological preparations, was unsuitable in this instance.

Actinophrys was examined in such preparations with a Zeiss research microscope (Standard WL) with attachments for phase-contrast, Nomarski interference-contrast or qualitative interference (colour microscopy). The lengths of axopodia were assessed using an eyepiece micrometer or by measurement from photographs of known magnification. The microscope was equipped with an electronic microflash device (Zeiss Ukatron UN 60) and a Zeiss attachment camera loaded with Kodak Panatomic X film. Kodacolor-X film was used for colour photography.

(e) Micromanipulation

Microelectrode glass tubing was first drawn out into a narrow thread by hand, and then pulled out to a very much finer point (tip diameter approx. 2 µm) using a De Fonbrune microforge. These fine glass needles were mounted in a De Fonbrune micromanipulator and used to bend axopodia. The manipulations were observed with a Zeiss inverted phase-contrast microscope (Plate 41). Actinophrys were contained in small chambers of the type devised by Gall (1954). These were made by fixing a coverslip over the bottom of a hole bored in a slide. The coverslip was held in place with embedding wax (Fig. 2).

Axopodia were damaged experimentally by drawing animals into, and expelling them from, a narrow glass pipette several times. Best results were achieved when the bore of the pipette was slightly greater than the diameter of the Actinophrys.

(f) Ultra-violet microbeam irradiation

Organisms were irradiated with an ultra-violet microbeam apparatus (Starling: in preparation). A mercury vapour lamp (Zeiss Jena 220/HB050) generating ultra-violet light of various wavelengths was used as a source.

This passed a beam of light into the intermediate tube of a Zeiss WL microscope perpendicular to the optical axis. When it reached the optical axis it was reflected downwards by a half-silvered mirror. The cross-sectional area of the beam could be varied. It was controlled by moving two sliding L-shaped plates (Fig. 3). The axopodium to be irradiated was positioned before exposure by moving the microscope stage controls until it was under the centre of the cross formed by the two closed jaws of the sliding plates. In these experiments the beam of ultra-violet light was focussed down to a square of side 26 μm by a x 32 Ultrafluar objective. The radiation intensity of the microbeam was of the order of 10^{-3} ergs/μm^2/sec. The surface area of the side of the axopodium exposed to the incident radiation was about 83 μm^2; hence the dose of radiation each axopodium received was approximately 8.3×10^{-2} ergs/sec. This figure only represents the incident radiation intensity, which may not correspond to the amount of radiation absorbed by the axopodium (Dendy and Smith, 1964).

When an axopodium situated fractionally below the coverslip was brought into focus a reflected image of the microbeam in the object plane was observed. This image was a blue light reflection from the coverslip culture medium interface. This situation, where the object to be irradiated and an image of the microbeam were both visible during the time of exposure, was only achieved with extremely thin hanging drops where the meniscus held the organism up close to the coverslip. Such hanging drops rapidly dried out through evaporation so extra drops of culture medium were placed in the cell to increase the humidity (Fig. 4). Organisms in preparations of this type sealed with silicone grease often survived for 5 hr.

(g) Low temperature: treatments and measurements

Organisms were maintained at various low temperatures (see Table II).

FIGURE 3

Movement of Plates 1 and 2 along the diagonal axis from bottom left to top right increases or decreases the cross-sectional area of the ultra-violet microbeam.

FIGURE 4

Illustrates the type of preparation used for ultra-violet microbeam irradiation studies. Actinophrys are contained in the hanging drop. The extra drops of culture medium in the well of the depression slide increase the humidity of the chamber and prevent large reductions in the volume of the hanging drop through loss of medium by evaporation.

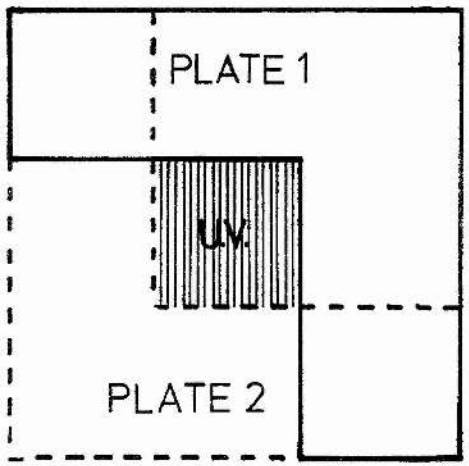
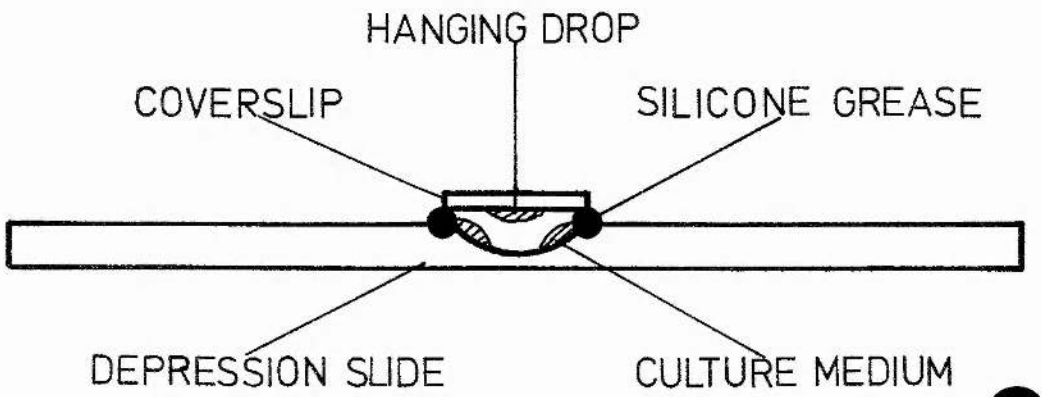


PLATE 1
U.V.
PLATE 2

3


HANGING DROP
COVERSLIP
SILICONE GREASE
DEPRESSION SLIDE
CULTURE MEDIUM

4

TABLE II: Low temperature treatments

Temperature	Container	Method of cooling	Experiment
15°C	Small culture dish	Gallenkamp cooled incubator	The effect of long-term cooling of axopodia
4°C	Ring preparations	Constant temperature room	The effect of low temperature on cytokinesis
0°C	Ring preparations	Crushed ice in an expanded-polystyrene container	The rate of recovery of axopodia after cold treatment
-2.5°C	Small culture dish	Gallenkamp cooled incubator	Electron microscopy of axonemes regenerating after cold treatment

The variation in temperature occurring in ring preparations, small cultures and small volumes of glutaraldehyde fixative was monitored with a thermocouple constructed using 4×10^{-3} in diameter wire. The junctions were prepared using copper and a nickel-chromium alloy (Eureka wire). The thermo-electric current was monitored either as a continuous record on a strip-chart recorder (Associated Electrical Industries) or by taking readings at timed intervals from a flying spot galvanometer (Pye Unicam). In both situations the instruments were calibrated against a mercury thermometer immediately before each series of measurements was made and checked again afterwards.

(h) Chemical treatments

Using light microscopical examination only, the effect of several chemicals on Actinophrys was tested (see Table III). When cytochalasin B treatments are made (Carter, 1967) the substance is used as a dispersion rather than a solution; even in some of the lower concentrations used here suspended particles were clearly visible with the microscope (App. 2).

TABLE III: The effect of certain chemicals

Chemical	Source	Concentration	Solvent
Colchicine	Calbiochem	0.75%	Culture medium
"	British Drug Houses	0.5%	" "
E.G.T.A.	" "	0.05-0.2%	" "
Cytochalasin B	I.C.I.	0.1-50 µgm/ml	Culture medium + Dimethyl sulphoxide (final conc. Dimethyl sulphoxide 0.1%)

(i) Transmission electron microscopy

Actinophrys at particular developmental stages were selected from cultures using a Zeiss stereo-binocular dissecting microscope and isolated by hand with finely drawn-out glass pipettes. They were then fixed by one of the two methods outlined below.

Method 1 (Tucker, 1967):

Organisms were fixed for 30 mins at room temperature with a solution of 2.5% glutaraldehyde (Sabatini et al., 1963) and 0.6% sucrose (Caulfield, 1957) dissolved in a 2×10^{-2} molar phosphate buffer (pH 7.8). They were then washed for 12 hr in several changes of a 2% sucrose solution and dissolved in the phosphate buffer. Fixation followed in a solution of OsO_4 (1%) and sucrose (4%) dissolved in phosphate buffer. The Actinophrys were rinsed again in the washing solution described earlier and then dehydrated and embedded.

Method 2 (Roth et al., 1970):

This technique, designed specifically for the preservation of heliozoans, gave superior general fixation to that of Method 1. Unless otherwise stated, electron micrographs are of material treated in this way.

A short prefixation (30 sec) was carried out by adding an equal volume of culture medium containing Actinophrys to a solution of

glutaraldehyde (12%), $MgSO_4$ (2×10^{-5} Molar), and sucrose (2×10^{-3} Molar) in a 3×10^{-2} Molar phosphate buffer. Variation of the pH of the buffer within the range 6.9-7.6 had no noticeable effect on the preservation. To this mixture of culture medium and glutaraldehyde was added an equal volume of a solution of OsO_4 (1%), $MgSO_4$ (10^{-5} Molar), and sucrose (10^{-3} Molar) in phosphate buffer (1.5×10^{-2} Molar). For 20-30 min the Actinophrys remained in this mixture which contained solutes at the following final concentrations:

Glutaraldehyde	3%
Osmium tetroxide	0.5%
Magnesium sulphate	10^{-5} Molar
Sucrose	10^{-3} Molar
Phosphate buffer	1.5×10^{-2} Molar

Actinophrys treated in either of these two ways were embedded in 2% agar plaques (approximately 1 cm in diameter) using the technique of de Haller et al. (1961). Each agar plaque served to support and protect the axopodia of approximately 30 organisms especially at the stages, from dehydration onwards, when they were most brittle. The agar plaques were handled in the same way as a small piece of tissue. They were dehydrated with ethanol and then immersed in 1.2 epoxy propane (3 changes, 30 min each). Flat embedding was accomplished with Araldite using the methods described by Luft (1961) and Borysko (1956). The agar plaques were treated with an epoxy propane/Araldite mixture (2 part:1 part, respectively) for 3 hours. A further 3-hour treatment was given in which the ratio of epoxy propane to Araldite was reversed. This process facilitated the penetration of the Araldite embedding medium. The plaques were then placed in the final embedding mixture which consisted of 27:23:1 parts by volume of Araldite, hardener, and accelerator, respectively.

A slight adaptation of the above procedure was used during the fixation and embedding of cysts which were often damaged when pipettes were used to lift them free from the bottom of Petri dishes. In this

instance the cysts were fixed *in situ*, still attached to the bottom of the dish. After fixation a thin layer of 2% agar was poured over the cysts. Early in the dehydration process this agar shrank around the cysts. The agar was then peeled free from the dish taking the cysts with it. The layer of agar was then cut into smaller pieces (squares of side about 2 cm); this facilitated the penetration of the embedding resin. The agar squares were then treated as described above.

After embedding, individual organisms at the required developmental stage were selected for sectioning by inspection with a phase-contrast microscope. After reorientation, if this was necessary, blocks were mounted on Araldite pegs and then trimmed. Final trimming of the face of the block was carried out with glass knives on an L.K.B. Ultratome III by cutting "thick" (1 µm) sections. These were stained with Methylene Blue (Mullinger, 1964) and examined using bright field illumination. Under these conditions the nuclei and two distinct layers of cytoplasm could be distinguished. These were used as indicators of position within the cell for the thin sectioning that followed.

Thin sections were picked up on copper grids coated with Formvar and evaporated carbon films. Sections were double stained with uranyl acetate (Gibbons and Grimstone, 1960) for 90 min followed by lead citrate (Reynolds, 1963) for 5 min. Sections were viewed in a Siemens Elmiskop I operated at 60 or 80 kV with a 50 µm objective aperture. The specimen chamber was cooled with liquid nitrogen by means of a "cold finger". Electron micrographs were taken on Ilford EM4 plates at magnifications between 2,000 and 40,000 times.

A densitometer (Joyce Loebl, Chromoscan) was used to scan positive transparencies prepared from plate negatives of sections through nuclear envelopes.

(j) Scanning electron microscopy

Glass coverslips were placed on the bottom of a Petri dish containing a month-old-starving-culture. When Actinophrys had settled and encysted the coverslips were taken and fixed in a 6% glutaraldehyde solution (see §i above) for 1 hr. To remove the outer jelly-like coating over the cysts they were treated for 10 min with dilute sulphuric acid. Following this they were dehydrated in a graded ethanol series (10 min each solution). The ethanol was replaced by diethyl ether in 4 stages during which the ratio of the 2 solutions was 1:3, 2:2, 3:1 and 4:0 respectively. The cysts were then left to air-dry in the presence of a dessicant (P_2O_5) for 24 hr. The upper surface of the coverslips bearing the cysts was coated with two consecutive layers; the first (carbon) was approximately 20 nm thick and the second (40% gold-palladium) was 30 nm thick (Johnson Matthey Metals, Ltd., Hatton Garden, London). The gold-palladium was evaporated from a tungsten filament in a vacuum coating unit (Edwards High Vacuum Ltd.) at between 5×10^{-4} and 5×10^{-5} torr (Harris et al., 1972). The coverslips were fixed to aluminium stubs with Uhu glue and the top surface was earthed to the stub with a small drop of conducting paint (M154 colloidal silver, Polaron, London). Specimens were viewed at accelerating voltages of 15 kV with a scanning speed of 50/sec in a Stereoscan 600 (Cambridge Scientific Instruments, Ltd., England). Scanning electron micrographs were made on Ilford FP4 film.

CHAPTER I

THE STRUCTURE OF *ACTINOPHRYS SOL*

Introduction

This account gives a concise description of the structure of the encysted and trophic forms of the interfission organism. Many of the light-microscope observations presented are confirmation of work performed by Belar (1923). Since his work was completed without the advantage of phase-contrast and Nomarski interference-contrast optics the description presented confirms and extends his observations.

Short papers by Kitching and his colleagues (Kitching, 1964; Macdonald and Kitching, 1967) in the 1960s first described the fine structure of *Actinophrys* and revealed the microtubular composition of the axial rods (the axonemes) which support the organism's radiating axopodia. The present study is more complete, and reveals greater detail, than Kitching's studies, probably as a result of improvements to electron microscope technique which have taken place over the decade.

This is not an exhaustive structural study; it is a necessary introduction to the developmental and experimental work which follows.

Results

(a) General organisation of trophic *Actinophrys*

The structure of trophic *Actinophrys* seen with the compound microscope is simple and has been well documented (Belar, 1923). The cell body is spherical (about 50 μm in diameter) and contains a single central

nucleus (about 10 µm in diameter). The cytoplasm is divided into concentric layers; the endoplasm appears more compact than the peripheral ectoplasm which is highly vacuolated. The surface of the cell often bears a superficial bleb (frontispiece), the contractile vacuole.

Projecting radially from the cell are numerous thin, finely-tapering axopodia of varying lengths (Plate 1). Some axopodia exceed 150 µm in length and longer axopodia usually have greater diameters at their bases than short ones. Even in the case of longer axopodia the diameter at the base rarely exceeds 3 µm.

Certain observations (Chapter VI) show that the thicker basal portions of axopodia are more resistant to certain physical and chemical treatments than the distal parts. The axopodia of Echinosphærium (Roth and Shigenaka, 1970) are similar to those of Actinophrys in this respect.

Each axopodium contains a central rod called an axoneme. Axonemes extend down into the cell body and terminate at the nucleus.

(b) Fine structure of trophic Actinophrys

Transverse sections through axopodia reveal the "double polygonal spiral arrangement" of microtubules (Harris, 1970) which compose the axonemes (Plates 4,6). This pattern of microtubules has been demonstrated previously in Actinophrys and Echinosphærium (Kitching, 1964; Tilney and Porter, 1965) but is not invariably present (Plate 7). The axonemes are divided into 12 sectors (Plate 4). Individual microtubules in one turn of the spiral may be linked to two, or four, other tubules by long cross-links and to two other tubules by short cross-links (Fig. 5). Cross-links between tubules in adjacent turns of the spiral are always long. Cross-links between adjacent tubules in the same turn of the spiral are always short (Plate 4). These short cross-links may be a double structure which can be resolved into two closely adjacent links (Roth et al., 1970).

Both types of cross-link are either absent or indistinct in axonemes

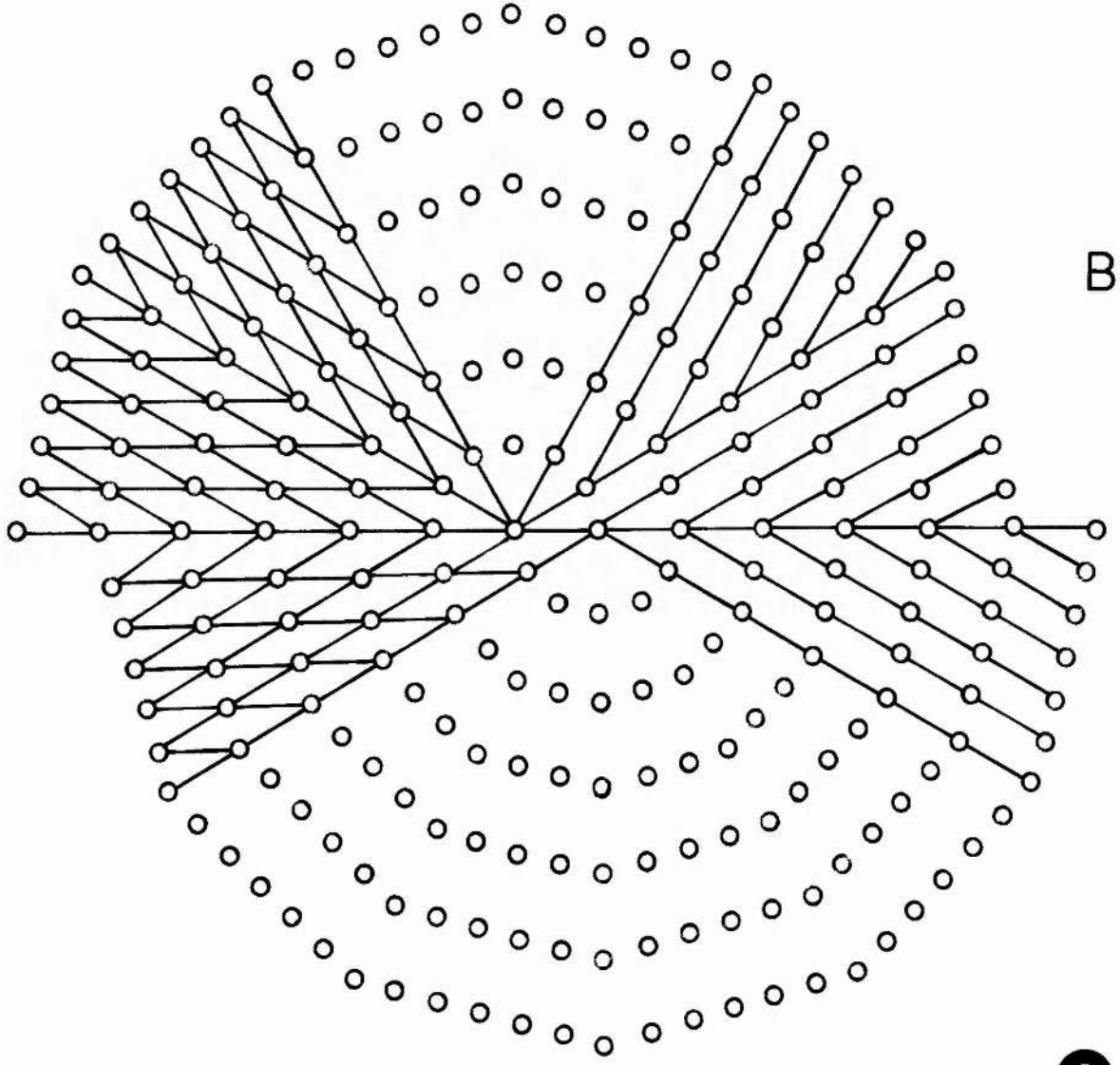

A
B
axis of
symmetry

FIGURE 5

A diagram of a transverse section through an axoneme. For convenience only long cross-links between microtubules are shown. Short cross-links (not shown) lie between adjacent microtubules in the same turn of each spiral. The axoneme is divided into 12 sectors. 3 of these sectors (A) illustrate the arrangement of long cross-links proposed on theoretical grounds by Harris (1970). According to the electron microscopists McDonald and Kitching (1967) and Tilney and Byers (1969) the pattern is that shown in the 3 sectors labelled B.

of organisms fixed by Method 1. The pattern of axonemes treated in this way is also less precise than that of axonemes fixed by Method 2. This is consistent with the hypothesis that the pattern of microtubules in the axoneme of Echinosphærium is defined by the position of the cross-links (Tilney and Byers, 1969). Transverse sections of axonemes fixed by Method 1 contain many C-shaped profiles where microtubules appear to have split open (Plate 5).

Axonemes attach at their bases to the nuclear envelope (Kitching and Craggs, 1965). No structural modification is resolved in the nuclear envelope localised at the site of axoneme attachment. The nuclear envelope is flattened where axonemal microtubules terminate on it with their ends precisely in register with one another (Plate 2). The nuclear envelope has a stepped configuration (Plate 8) in a few cases where one group of tubules in an axoneme is displaced towards the centre of the organism with respect to the other.

Apart from large axonemes which often contain more than 200 microtubules, there are numerous smaller groupings of tubules which are arranged in a similar pattern (Plate 12). These small axonemes are not always long enough to project into axopodia but often appear to be attached at their bases to the nuclear envelope. It is possible that all axonemes are attached to the nuclear envelope. Single tubules, small groups of tubules, and axonemes, are almost invariably radially oriented (Plate 12).

In contrast to the finding of Tilney and Porter (1965), who examined Echinosphærium, it appears that the bilateral axes of symmetry of transverse sections through adjacent axonemes are not always parallel in Actinophrys (Plate 12). Although axonemes and axopodia seem to be fairly evenly distributed, there is no obvious overall pattern or regular spacing of them.

When sections are cut perpendicular to the surface of the nuclei of

organisms fixed by Method 1 an unusual substructure is revealed (Plate 3). The nuclear envelope is of a type which apparently has never previously been described. It differs from other nuclear envelopes because it contains two extra electron-dense layers. Optical densitometer scans across highly magnified positive transparencies demonstrate this convincingly; they also show that the two inner layers stain less densely than the two outer layers (Fig. 6). Nuclear pores are found commonly in the envelope and usually appear to be filled with densely staining material (Plate 3). At the edges of the pores, where the two outer layers of the envelope come together, the two inner layers also appear to fuse. This frequently gives the impression that there is an envelope within an envelope. A broad electron-dense band of material lies within the nucleus adjacent to the nuclear envelope (Plates 67, 70 and 73). Anderson and Beams (1960) have discussed the composition of a band in a similar position in the nuclei of Echinosphaerium. They suggested that it was composed of "multi-nucleoli".

The endoplasm contains several types of membrane-bounded vesicles. These can be electron-lucent or may contain faintly staining amorphous material (Plate 12). One type of these vesicles occurs throughout the cytoplasm. They contain greater amounts of dense material the nearer they are to the cell surface. Vesicles of this type at the cell surface are termed haptocysts (Plates 6, 7).

Coated vesicles (Plate 12) are a common component of the endoplasm; apparently they are occasionally connected to Golgi bodies. This observation is consistent either with the theory that coated vesicles are transporting proteases to food vacuoles, or that they are segregating material for transport to the forming haptocysts (see Tilney and Porter, 1965).

Lipid droplets which are not membrane-bounded are occasionally present. They lie, as Kitching (1964) has stated, at the level of the

FIGURE 7

Illustrates the life cycle of *Actinophrys*. The sexual and resting phases take place within a cyst which is not shown. No estimate is given for the duration of the colonial phase of the life cycle as it appears to be extremely variable.

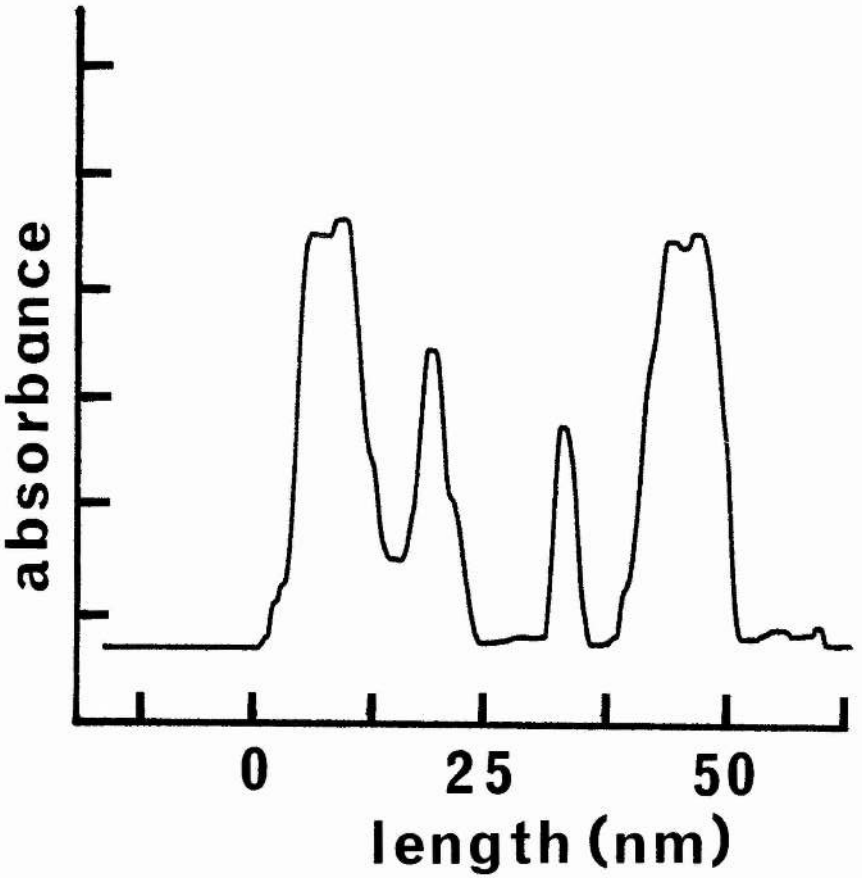
junction between the endoplasm and ectoplasm. Axonemal vesicles (Roth et al., 1970) are commonly found clumped near axonemal microtubules; they are roughly spherical bodies about 80 nm in diameter, which appear to be formed from tightly folded membranes or filaments (Plates 63, 67). In the event that they are membranes, they differ from normal unit membranes because only a single dense layer is sometimes present.

Large electron-lucent vacuoles (Fig. 76), which are bounded by unit membranes, occupy most of the ectoplasm. Mitochondria with tubular cristae are situated throughout the ectoplasm in the thin strands of cytoplasm between the large vacuoles. They are also found in axopodia (Plate 84) and in the band of endoplasm which borders the ectoplasm (Plate 70). Mitochondria do not occur in a broad endoplasmic layer adjacent to the nucleus (Plate 12).

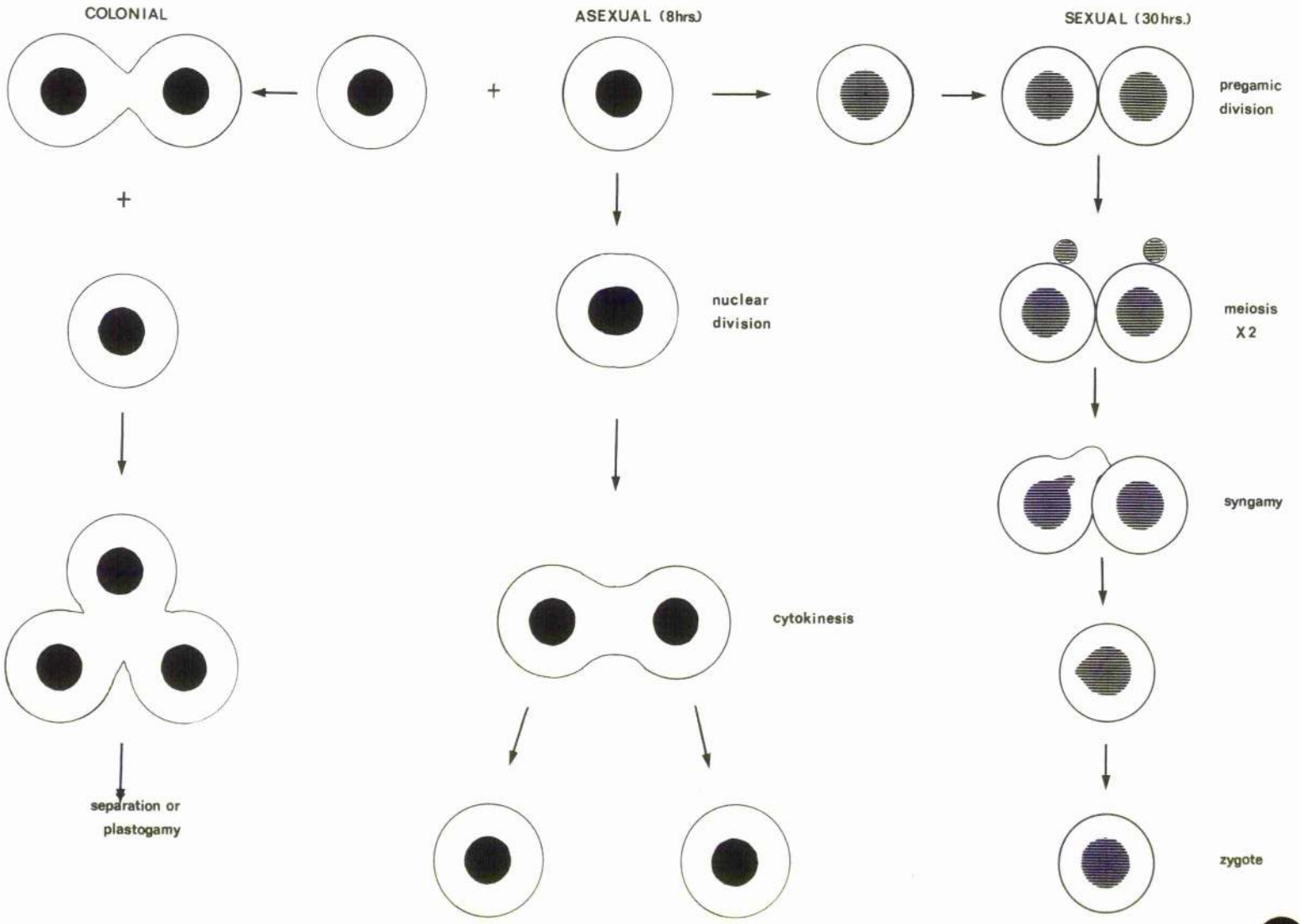
Haptocysts are about 0.5 µm in diameter. They contain densely staining material, are found in large numbers at the surface of the cell, and in the axopodia (Plate 6). The dense granules within the vesicles are variable in shape, and often fill only half of each vesicle. The haptocyst vesicles are probably involved in food capture, because when they are ruptured by fixation the dense material is expelled and appears to become a flocculent mass. This flocculent material could form the thin strands seen with the light microscope which cause struggling prey organisms to adhere to axopodia. These vesicles are apparently analogous with the haptocysts of centrohelidans, another heliozoan family (Bardele, 1970). However, unlike the haptocysts of the centrohelidan Acanthocystis (Roby, 1972) the vesicles in Actinophrys have no visible substructure.

(c) Encysted Actinophrys

The sexual and resting phases of the life-cycle of Actinophrys (Fig. 7) take place within a cyst, which is roughly spherical (about 40 µm in diameter), and is often attached to the bases of culture dishes by a


absorbance
0
25
50
length (nm)

LIFE CYCLE OF ACTINOPHRYS SOL (EHRENBERG)



transparent gelatinous material (Belar, 1923) (Plate 11). The parts of the cyst which represent the cell body of the trophic organism are still recognisable within four concentric layers of material. In order, starting with the outermost layer, these are the outer layer, the intermediate zone, the outer zygote wall, and the inner zygote wall (Fig. 8).

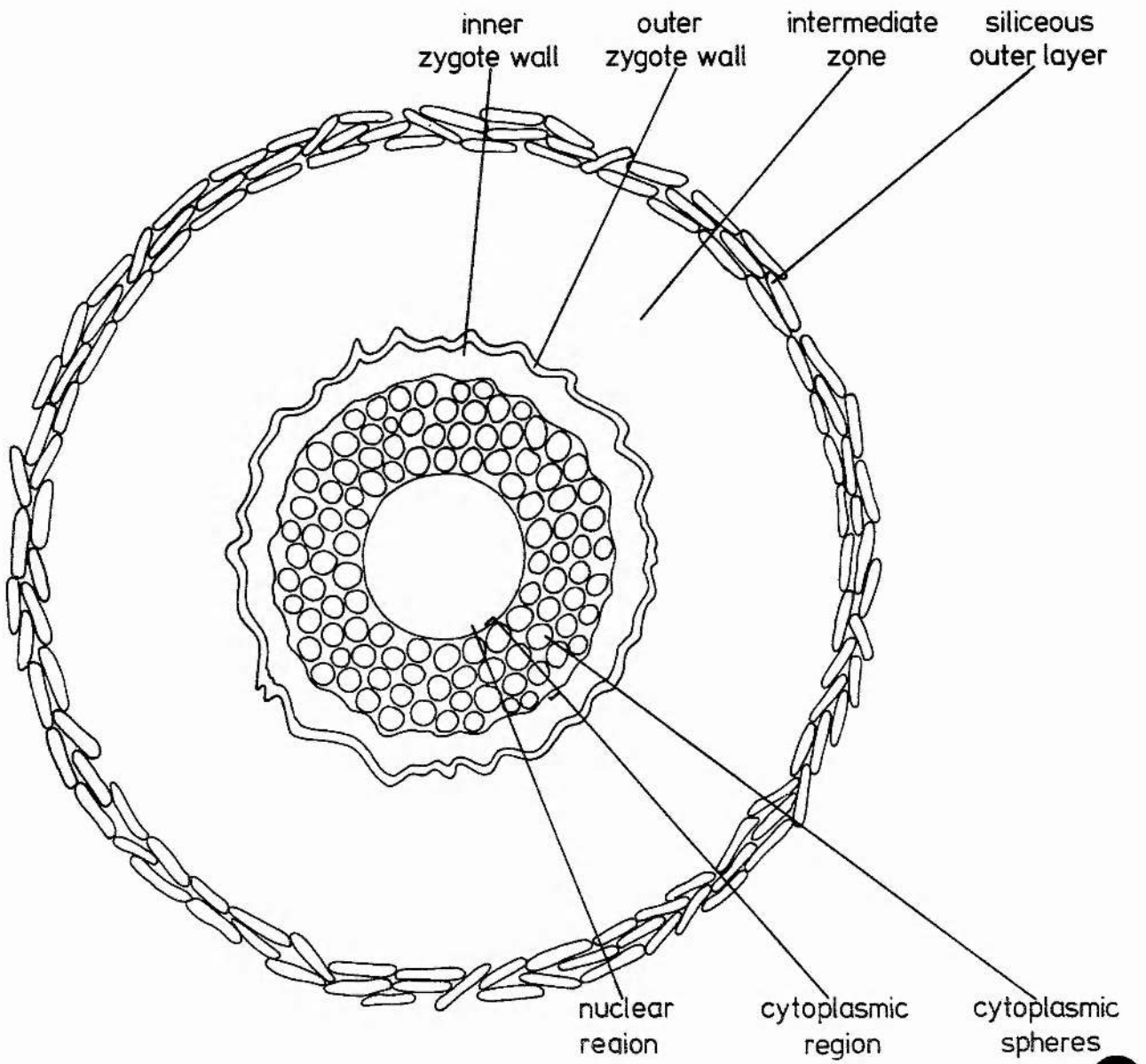
The outer layer is about 2 μm thick and is highly refractile. It appears to be constructed from small flat plates. The longest dimension of these plates is about 2 μm; they are about 0.5 μm thick. They lie 2 or 3 deep in a layer overlapping each other like haphazardly placed tiles. Scanning electron micrographs show that the flat plates have irregularly shaped edges (Plate 10). The outer layer is resistant to prolonged (24 hr) treatment with 50% sulphuric acid; it is probably siliceous. This interpretation is supported by the brittle nature of the material which often appears to have shattered during sectioning (Plate 9).

The intermediate zone is about 10 μm thick. It is transparent when examined with both the light and electron microscopes. It is crossed by a small number of strands of dense material (0.1 μm thick) which appear to bind the outer layer to the outer zygote wall. These strands may therefore give support to the outer layer. Two layers make up the zygote wall. Together they are about 1 μm thick. The outer zygote wall is slightly less dense than the inner zygote wall (Plate 9).

The cell body, which represents the contents of the copulation cyst described by Belar (1923), can be resolved into an outer cytoplasmic region and an inner nuclear region. The nuclear region is roughly spherical (about 10 μm in diameter), homogeneous, and of medium density. No nuclear envelope can be distinguished. The cytoplasmic region is "packed" with small spheres (about 1 μm in diameter). Each has a central region (0.5 μm in diameter) which stains less densely than its periphery. These spheres are set in a matrix which contains electron-transparent regions (about

FIGURE 8

A diagram of a section through an encysted Actinophrys. The section is parallel to the plane of the surface to which the cyst was attached at the level of the cyst's nuclear region.


inner
zygote wall
outer
zygote wall
intermediate
zone
siliceous
outer layer
nuclear
region
cytoplasmic
region
cytoplasmic
spheres

100 nm in diameter) which are most common adjacent to the small spheres and the zygote wall (Plate 9).

Discussion

(a) Axonemal pattern

The pattern of axonemal microtubules and cross-links in Actinophrys and Echinosphærium is apparently identical. The highly ordered nature of the array has attracted much attention, and many authors have attempted to describe and explain the formation of the system as a "crystallisation" in self-assembly terms (Porter, 1966; Tilney and Byers, 1969). The pattern of long cross-links proposed on theoretical grounds by Harris (1970) is different from the pattern suggested by electron microscopists (Fig. 5). According to McDonald and Kitching (1967), and Tilney and Byers (1969), only those tubules at sector interfaces (Fig. 5) bear two long links which attach to tubules in the next outermost spiral. Conformations such as those found in Actinophrys (Plate 4) and Echinosphærium (Roth et al., 1970; Fig. 5) are therefore expected according to earlier theories because the tubules involved are placed at sector interfaces. Similar V-shaped profiles, of two long cross-links, should be attached to every microtubule according to Harris's (1970) model. However, microscopy indicates that these conformations are never found attached to microtubules situated within sectors.

Harris (1970) questions the existence of the short links originally postulated by McDonald and Kitching (1967). He does so on the grounds that their presence is apparently not required to maintain the structural integrity of the axoneme. However, their appearance in recent micrographs is quite consistent, and the morphological evidence for their existence is at least as strong as it is for the long links (Roth et al.,

1970). One possibility that has not been considered is that the short links are required in a process of semi-conservative replication of axonemes and their pattern. It is known that new axonemes are formed because succeeding generations of Actinophrys bear as many axopodia as their predecessors. However, to date, nothing is known of the way in which this occurs. The process of axoneme replication might proceed in the following way. An unspecified substance may break down long cross-links. If the short links are not sensitive, or are much more resistant to its action so that they remain when the long links are destroyed, the two spirals of the axoneme would be effectively uncoupled. In certain cases, random or non-random movements could separate the two spirals. Each of the separated spirals could then act as a template for the accumulation of newly formed or forming microtubules when suitable conditions for long-link formation return. In this way two new axonemes would be formed.

Certain observations support this theory. Because the structure of the two types of link is different their comparative resistance to various damaging influences is also likely to be different. The suggestion that only particular parts of axonemes may be broken down is not unreasonable because certain results (Chapter III) show that restricted parts of the axonemes of Actinophrys are broken down at a stage prior to cell division. A similar situation has been reported where old microtubular feeding organelles are broken down in the ciliate Nassula (Tucker, 1970). In these two cases it is not clear whether both the microtubules and the cross-links, or just the cross-links, are broken down. Whichever proves to be the case there seems little doubt that structural proteins are often broken down at specific times and at restricted sites.

This theory for axoneme replication indicates a function for the short links, namely maintenance of the integrity of single spirals whilst they are separate. One obvious prediction which can be made from this

theory is that small groups of tubules should exist which have a single row of microtubules. Such groupings occur in Actinophrys (Plate 12) but have so far not been reported for other heliozoans.

(b) C-microtubules

Do C-microtubules present artefacts produced by the splitting of microtubule walls during fixation, or do they represent a real phase in naturally occurring breakdown or assembly of microtubules (Cohen and Gottlieb, 1971)? The presence of C-microtubules in axonemes after fixation by Method 1, whereas they are absent after fixation by Method 2, suggests that the C-shaped profile is an artefact produced at the time of fixation.

It is of interest that the split appears to be a single longitudinal one. This finding indicates that the bonds between adjacent subfibres in the tubule wall is in some way weaker than the bond between adjacent subunits making up the subfibres. It does not necessarily follow that the formation of this weaker bond is the final stage in normal development of the tubule.

(c) Nuclear envelope

The unusual structure of the nuclear envelope may be related to its unusual function. It bears the relatively very long and rigid axonemes which themselves hold the external cell membrane in its characteristic shape. Therefore it seems likely that at least part of the role of the extra layers is one of structural reinforcement. In their review, Stevens and André (1969) discuss the role of various constituents present in nuclear envelopes. One common structure is the thickened internal lamella. Fawcett (1966) has suggested that this lamella has a supporting function. It is worth noting that this internal lamella which is found in an "exaggerated" form in the "honeycomb layer" of Amoeba proteus (Gall, 1964) is absent in Actinophrys.

It has occasionally been suggested that parts of the nuclear envelope have a synthetic role. Mastigonemes (components of flagellae) appear to be formed in perinuclear cisternae (dilations of the nuclear envelope) in the flagellate Ochromonas (Bouck, 1971). These mastigonemes are pinched off in large "outpocketings" from the outer nuclear membrane, and are then transported to the cell surface. The absence of such "outpocketings" in Actinophrys suggests that if the extra layers in the nuclear envelope are a synthetic product then they are transferred to the required site by a different process from that used in Ochromonas.

Foci (Porter, 1966), orienting centres (Inoué and Sato, 1967), nucleating centres (Tilney, 1968), microtubule orienting centres (Pickett-Heaps, 1969) and initiating sites (Tucker, 1970), are similar hypothetical structures. They are all held to be responsible for aspects of the spatial arrangement and the directional orientation of microtubules in various situations. Is the pattern or orientation of axonemal microtubules in Actinophrys defined by such sites at specific places on the nuclear envelope? The "stepped configurations"(Plate 8) in nuclear envelopes indicate that this is unlikely. At one point tubules are attached laterally to the nuclear envelope, at another they are attached terminally. This seems to rule out orientation of microtubules as a function of the nuclear envelope. The "stepped configuration" also presents difficulties if it is assumed that the nuclear envelope determines axonemal pattern. A pattern of nucleating sites fixed on the envelope would not give rise to a normal axoneme if the envelope was folded in this manner. A more reasonable interpretation of the situation is that the nuclear envelope has a "bonding affinity" for microtubules. That this affinity can be for both the sides and ends of tubules is a feature of considerable importance.

If the axes of bilateral symmetry of neighbouring axonemes are parallel it might indicate that the relative positioning of axonemes is

maintained by a pattern of attachment or nucleating sites on the nuclear envelope. However, as I have shown, there is no evidence for a parallel relationship between the bilateral axes of symmetry of neighbouring axonemes in Actinophrys (Plate 12). Comparison of the evidence of Roth et al. (1970) for Echinosphærium with Tilney and Porter's (1965) statement that the axonemal axes of symmetry are parallel leads to the conclusion that on this point Tilney and Porter are in error.

(d) Haptocysts

A progression of vesicles of increasing density can be traced from deep in the endoplasm to the cell surface (Plates 76, 77). This succession of vesicles may represent stages in the development of the haptocysts (Bardele, 1970). The fact that these vesicles originate in the region of the Golgi apparatus, and Kitching's (1960) observation of their metachromacy with toluidine blue staining, are consistent with the suggestion that most of the materials in the haptocysts are acid mucopolysaccharides.

(e) Axonemal vesicles

These bodies may be composed of, or contain, some material which is necessary for the formation of axonemes. This material could be tubulin or equally well an as yet uncharacterised material, such as for example cross-link material or some material from the lumen of the tubules or clear zone surrounding them. Evidence for the biochemical composition of these axonemal vesicles might be gained by using already established techniques. One obvious approach is the application of ^{3}H-colchicine (which binds tubulin), either in combination with electron microscope autoradiography, or with fractionation experiments monitored by electron microscopy and liquid scintillation counting (Borisy, 1972). Evidence in favour of the theory that these vesicles contain material which forms part of the axonemes is not just restricted to the proximity of these bodies to

axonemes. In Chapter VI morphological evidence for an intimate association of vesicles with the ends of outgrowing microtubules is presented. This is complemented by the observation that there is an inverse relationship between the number of axonemal vesicles and the number of microtubules present when the organism recovers after cold treatment.

(f) The cyst

Unfortunately the quality of fixation of the cyst was so poor that it was not possible to ascertain whether the axonemal pattern survived through the sexual (encysted) phase of the life-cycle (Plate 9). Because the danger of producing artefacts is greater when the fixation is poor no attempt will be made to speculate about the nature of cyst components such as the enigmatic 1 μm diameter spheres (Plate 9). However, some sound information has been gained about the outer layer of the cyst.

This is not a complete layer and probably only protects the cell body against mechanical damage. The main osmotic barrier may lie in one or both of the zygote walls. It is probably this osmotic barrier which makes it difficult to achieve good fixation. Both the layers of the zygote wall are probably organic. One may be similar to the chitin-like "latticed capsule" of the related heliozoan Clathrulina elegans (Bardele, 1972).

CHAPTER II

BINARY FISSION

Introduction

This chapter is a record of several observations and experiments designed to further understanding of binary fission in Actinophrys; it attempts to relate the process to current theories of cell division.

An indication that binary fission in some rhizopods might involve an unusual type of cytokinesis comes from a study made by Chalkley (1935) on Amoeba proteus. He suggests that amoeboid movement is employed, and that daughter organisms divide by "walking apart".

Binary fission, in Actinophrys, has only been mentioned by two recent authors. Kitching (1964) states that there is no obvious activity of the axopodia. Watters (1968) has analysed the movements of Echinosphærium and Actinophrys; he reports that dividing organisms roll apart. Thus heliozoa and amoebae appear to employ their normal means of locomotion as a division mechanism. It is worth noting that all these organisms are rhizopods.

The "cortical gel" contraction theory of cytokinesis (Marsland and Landau, 1954) has gained much support from recent morphological studies which reveal a distinct ring which may be contractile. The initial discovery of this ring of actin-like microfilaments in the region of the advancing cleavage furrows of dividing Arbacia eggs (Schroeder, 1969) was followed by its demonstration in dividing Triturus eggs (Selman and Perry, 1970), and in dividing HeLa cells (Schroeder, 1970). It is possible that this is a widespread division mechanism. However, it is certainly

modified in at least one situation. Tucker (1971b) has convincingly demonstrated the ephemeral presence of a girdle of microtubules which lies parallel to the axis of cell separation and between the contractile ring and the pellicle during binary fission in the ciliate Nassula.

In some plants and animal cells (Buck and Tisdale, 1962; Pickett-Heaps, 1972) fusion of a number of vesicles in the division plane apparently accounts for the separation of the two daughter cells.

Results

(a) Binary fission — major structural changes

Many organisms from 4-day-old cultures contain two nuclei. These Actinophrys have completed nuclear division and are about to undertake cytokinesis. They are recognisable using a dissecting microscope because of their oblong shape. The description of binary fission in Actinophrys which follows is based on observations of dividing organisms in ring preparations. Unless otherwise stated these observations have all been made on Actinophrys which were floating freely in the culture medium, because organisms which adhere to the bases of Petri dishes are often damaged when they are sucked free into a glass pipette. Plates 13-20 show the division of an organism. As division proceeds the two cell nuclei gradually move apart. The bridge of cytoplasm which connects the putative daughter cells lengthens and attenuates. Frequently food vacuoles or other vacuoles occur in bridge regions (Plates 15, 23). Sometimes these vacuoles have greater diameters than the parts of the bridge to either side of them and frequently appear to completely occlude the bridge between the two cells. Finally the connecting bridge breaks. The two half-bridges bend downwards as they break, and are gradually resorbed into their respective cell bodies. Bridges do not remain rigidly extended. The length and diameter of the bridge between daughter

cells at the time of final separation varies from individual to individual. It is often as much as 100 μm in length and as little as 2 μm in diameter.

(b) Rate of division

Measurements made on photographs taken at intervals allow the rate at which dividing cells move apart to be calculated. Table IV indicates that the rate at which daughter organisms move apart is quite variable. During the 5 min period before bridge-breaking some cells move faster, and other cells move more slowly, than in the 5 min period after bridge-breaking.

TABLE IV: Rates at which dividing Actinophrys move apart ($\mu m\ min^{-1}$)

Organism	During 5 min before breakage of cytoplasmic bridge	During 5 min after breakage of cytoplasmic bridge
1	18.6	6.2
2	21.6	13.0
3	4.7	12.5

(c) Axopodial contacts

As dividing organisms move apart contacts are occasionally seen between two axopodia based on different cell bodies (Plates 24, 25). These are most frequent in the region between the dividing organisms. Axopodial contacts are of three types, terminal for both axopodia (double terminal), terminal for just one axopodium (single terminal), or not terminal for either (double non-terminal). These contacts are V, T, and X-shaped, respectively. Frequently a webbed region is visible at the point of contact between axopodia (Plate 21). Sometimes one or both of the contacting axopodia are bent (Plate 24). The degree of curvature of such axopodia sometimes varies as division proceeds. In the case of single terminal contacts the direction of the curve in the axopodium alters. Axopodia involved in double non-terminal contacts often move

with respect to each other in an incomplete, slow, "knitting motion".

Haptocysts continue to move up and down these contacting axopodia. Contact points do not block their progress, but haptocysts in one axopodium are apparently not able to cross over at the contact point and enter the other axopodium. Haptocysts sometimes move into the webbed regions, and such haptocysts often move back into the main axoneme-containing portions of axopodia. These observations indicate that the cytoplasm of the two contacting axopodia may be separated by membranes.

(d) Formation of new axopodia

Breakage of most axopodial contacts occurs rapidly. Axopodia just spring apart. Formation of contacts happens sufficiently rarely for it to have escaped detection. These contacts could possibly be formed by developing axopodia which sometimes grow out in the area to either side of the cytoplasmic bridge which connects dividing organisms. Small axopodia occur frequently in this region (Plate 22). Their lengths change continually. They alternate between periods of growth and resorption. Table V provides examples of the types of axopodial length changes which have been observed.

TABLE V: Length changes in axopodia in the furrow region

Time (min)	Axopodial length (μm)				
	Axopodium 1	Axopodium 2	Axopodium 3	Axopodium 4	Axopodium 5
0	12	7	12	10	21
2	12	15	19	10	30
4	30	15	24	12	24
6	24	15	19	15	22
8	29	12	15	15	22
10	34	15	10	19	15

The maximum elongation rate recorded for these axopodia is $4\ \mu m\ min^{-1}$; the maximum rate of shortening observed has about the same value. Longer growing axopodia have greater diameters at their bases than shorter

growing axopodia. Although axopodia nearest to the bridge of cytoplasm connecting the two cells tend to be shorter than those furthest from the bridge a precisely graded arrangement of axopodia in order of decreasing length is not apparent (Plate 22). The appearance of these axopodia which grow and are resorbed during this part of the life cycle is restricted to a localised area of the surface of the organism. Examination of the two half cell bodies further from the connecting bridge of cytoplasm over long periods (2 hr) fails to reveal any small growing axopodia at a time when growing axopodia are continually present near the connecting bridge of cytoplasm.

(e) Rolling movements and division

Examination of living oblong Actinophrys, which lie on the bottoms of Petri dishes, shows that as division proceeds the connecting bridge of cytoplasm between the daughter organisms rises slightly. This is consistent with the suggestion (Watters, 1968) that daughter organisms divide by rolling apart. Using finely pointed needles, to make scratches on the bases of Petri dishes adjacent to organisms at early division stages, an assessment can be made of the relative movement of daughter organisms as they separate. These studies show that sometimes one daughter rolls, at other times both roll away from each other.

In order to discover whether attachment to a solid substrate is necessary for cell separation single, free-floating, oblong Actinophrys were "tumbled and spun" in the culture medium with a fine glass needle. The movement of the organisms was observed continuously; organisms were never allowed to settle on the substrate. Usually an apparently normal division was completed within 15 to 30 min from the start of the manipulations. This experiment shows that Actinophrys are able to divide when they are not attached to any substrate.

(f) The connecting bridge of cytoplasm

Actinophrys in late division stages were selected for sectioning from material embedded for electron microscopy (Materials and Methods). Median longitudinal sections of cytoplasmic bridges passed through both nuclei of dividing organisms (Plate 28). These do not reveal rings of microfilaments or any microtubular structures in the bridges. It is pertinent that in these organisms microtubules are well preserved in both axopodia and cell bodies. Cytoplasm in the bridges has a highly vacuolated appearance (Plates 27, 28). The vacuoles are arranged so that no path of cytoplasm is available in which a straight rigid skeletal structure could run from one cell body to the other (Plate 28).

(g) Influence of cold, colchicine, and cytochalasin B on division

(i) Cold. Ring preparations containing about 40 oblong organisms were used in these experiments. Half of these preparations were maintained at room temperature (20°C), the other half were cooled to 4°C. After 6 hr, over 50% of the organisms at room temperature had divided but none of those at 4°C had done so. Half the cold treated Actinophrys were then allowed to warm to room temperature. After a further 6 hr over half of this group of organisms had divided. 24 hr after the start of each experiment, none of the Actinophrys still at 4°C had divided. Finally when these organisms were removed from the cold and allowed to warm to room temperature, about half of them divided within a further 5 hr. Thus, although after only 6 hr at 4°C division is completed faster, inhibition of division is still reversible in the majority of cases even after 12 hr at 4°C. The number of organisms which have divided in each of the groups at 1 hr intervals is shown in Fig. 9.

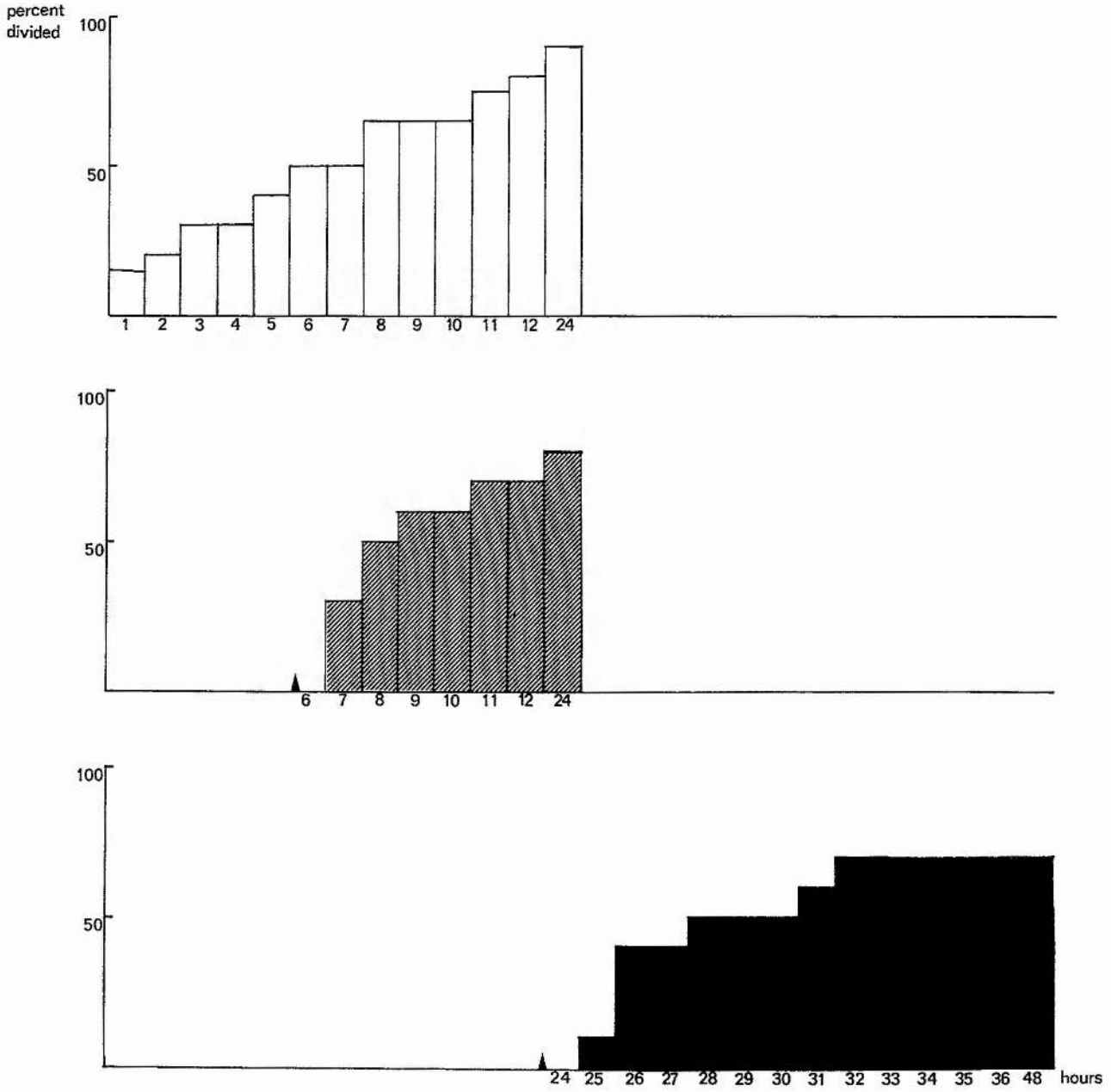
(ii) Colchicine. Similar experiments were performed using colchicine. The minimum concentration of colchicine in aqueous solution which causes visible shortening of axopodia in Actinophrys is 0.25%; 0.75% colchicine solutions were used in the following experiments which were

FIGURE 9

The first histogram (white bars) shows the percentage of organisms which have divided in a group maintained at room temperature.

The second histogram (shaded bars) shows the percentage of organisms which have divided in a similar group maintained at 4°C for 6 hr then placed at room temperature.

The third histogram (black bars) shows the percentage of organisms which have divided in a group maintained at 4°C for 24 hr then placed at room temperature. The division of organisms is totally inhibited at 4°C. This inhibition is reversible.


percent
divided
100
50
1 2 3 4 5 6 7 8 9 10 11 12 24
100
50
6 7 8 9 10 11 12 24
100
50
24 25 26 27 28 29 30 31 32 33 34 35 36 48
hours

conducted at room temperature.

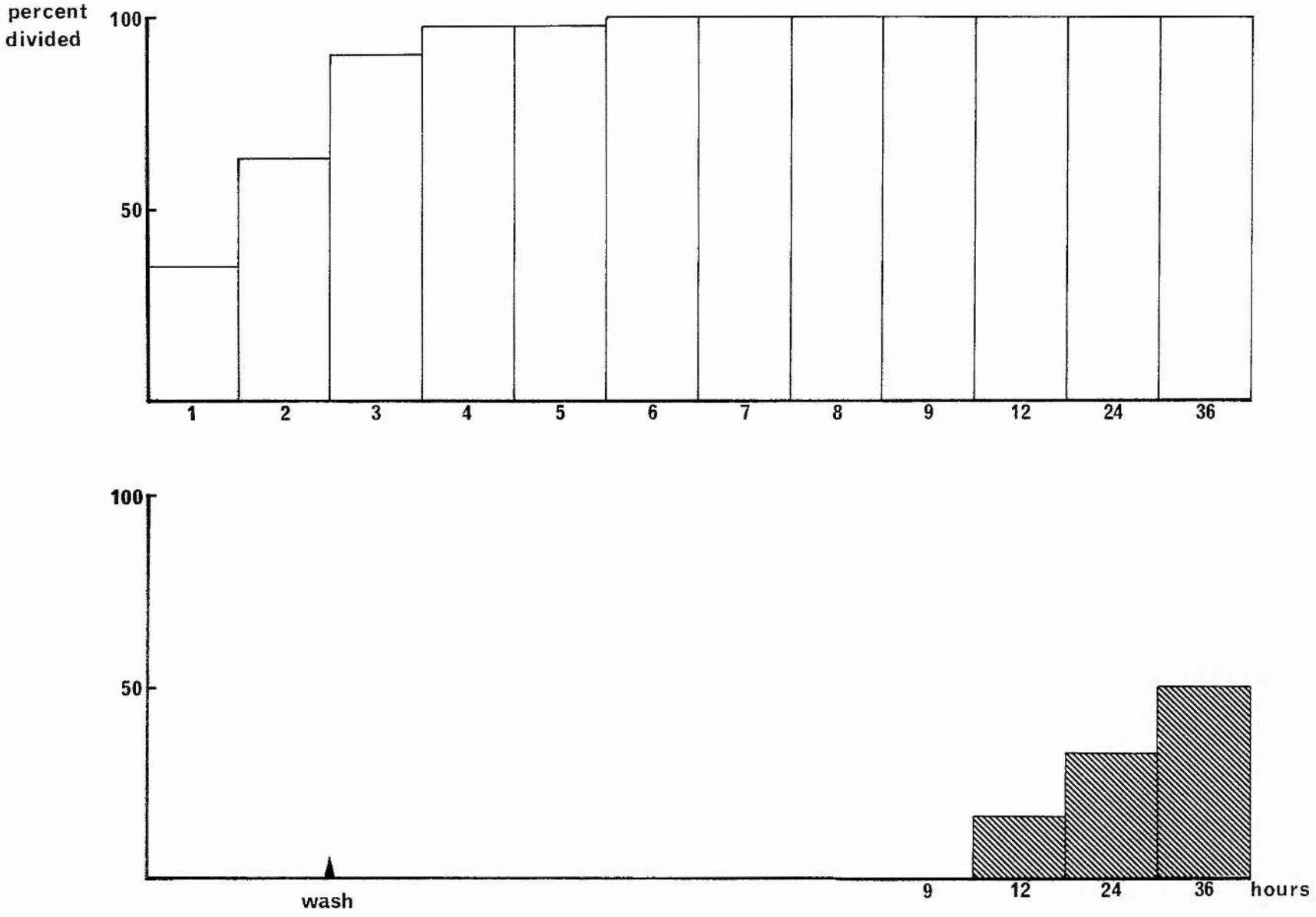
Oblong Actinophrys were added to 0.75% solutions of colchicine dissolved in culture medium and contained in small cylindrical "teflon" chambers (about 0.5 cm diameter) with transparent dialysis membranes forming their bases. Actinophrys in these chambers were observed with a dissecting microscope. Other chambers containing Actinophrys and culture medium with no colchicine were prepared. When half of the organisms in culture medium alone had divided, none of the colchicine treated organisms had done so. Most of the colchicine treated culture medium was then pipetted out of the chambers and replaced with fresh culture medium. Most of the colchicine that remained was removed by dialysis against a large volume of fresh culture medium for 1 hr. This was accomplished by placing the chamber onto a beaker containing a large volume of culture medium which was agitated with a magnetic stirrer. The treated organisms were then gently pipetted onto slides. Ring preparations were made and the progress of cell division was monitored hourly. The results are summarised in Figure 10. Within 36 hr half of the colchicine treated Actinophrys had divided. The percentage (about 50%) of cells which divide after this treatment is lower than the percentage (about 80%) which divides after 6 hr cold treatment. This lower incidence of division is not due to the colchicine remaining in the culture medium. The ultraviolet light absorption of the culture medium in the chambers after dialysis showed that colchicine was present at a concentration in the range .01-.001%. These concentrations are well below that (0.25%) which causes axopodial shortening.

(iii) Cytochalasin B. A number of oblong Actinophrys from 4-day-old cultures were placed in solid watch glasses which contained a 1% solution of dimethyl sulphoxide and concentrations of cytochalasin B to a maximum of 10^{μ}gm ml^{-1}. The numbers of organisms which had divided were monitored at 1 hr intervals. Division of binucleate Actinophrys was not

FIGURE 10

The first histogram (white bars) shows the percentage of organisms which have divided in a group maintained in culture medium at room temperature.

The second histogram (shaded bars) shows the percentage of organisms which have divided in a similar group treated with a solution of colchicine for 2 hr and then washed and placed in fresh culture medium. Colchicine inhibits division. This inhibition is reversible.


percent
divided
100
50
1
2
3
4
5
6
7
8
9
12
24
36
100
50
wash
9
12
24
36
hours

inhibited after 6 hr in the presence of a 10^{μ}gm ml^{-1} concentration of cytochalasin B dispersed in a solution of 0.1% dimethyl sulphoxide dissolved in culture medium.

(h) Colonial Actinophrys

One final item of information which is pertinent to the observations reported above is not based on observations of cells undertaking binary fission. It concerns a peculiar facet of heliozoan behaviour — the frequent aggregation of individuals into groups. This is thought to allow heliozoans to capture prey organisms which are too powerful to be caught by a single individual (Jepps, 1959). When a group of these organisms has digested its common meal the group splits up. The process by which individuals separate from groups is similar to certain events occurring during binary fission. Cytoplasmic bridges have similar shapes, and lengthen at similar rates, to the bridges between dividers at binary fission. The cytoplasmic bridges eventually snap separating individual organisms from clumps. Interestingly, there are bent axopodia between separating cells and clumps (Plate 26). These occur in similar places and are similar to the bent axopodia between Actinophrys undertaking binary fission.

Discussion

(a) Absence of a contractile ring

The popular contractile ring theory probably has no relevance to the division of Actinophrys for several reasons. First, no microfilamentous ring appears to be present. Negative evidence of this kind is not usually given much importance but in this case is more convincing than most. The fixation of the cell is adequate to preserve highly labile microtubules,

so it is likely that a ring of microfilaments would be preserved too, if one were present. Further, it would be very difficult to miss the postulated ring during sectioning of the material. The shape of the bridge is such that if it were produced by contracting microfilaments the ring would have to be very long indeed. In fact it would have to be a tube with a length of up to 100 μm in some cases. This is a huge "target" using presently available sectioning techniques.

Additional evidence for the opinion that there is no contractile ring in Actinophrys is provided by the finding that organisms continue to divide in the presence of cytochalasin B. Cytochalasin B is a mould metabolite which is known to inhibit cleavage of the newt egg, a process which is apparently mediated by the contraction of a ring of actin-like microfilaments (Perry, John and Thomas, 1971). Unfortunately this is a weaker argument than that based on the morphological evidence. The lack of inhibition of division in this instance could be explained by the drug not entering the organism. Many protozoa have low permeability to certain drugs. For example the lowest concentrations of colchicine which induce shortening of axopodia in Actinophrys are 5,000 times greater than the highest concentrations normally used to arrest mitosis in tissue culture cells (Priest, 1969). Although this evidence is inconclusive, it is well in accord with the morphological evidence which indicates that a "contractile ring" is not responsible for cytokinesis in Actinophrys.

(b) Division by locomotion?

Watters' (1968) suggestion that when Actinophrys divides the two daughter organisms move apart by rolling over the substrate explains many of the features observed during fission. It cannot explain the simple experiment which demonstrates that freely floating Actinophrys are capable of dividing. This latter observation indicates that floating organisms must "push against each other" when they are dividing for there is no

fixed object close enough to the cells such that a pulling or pushing force can be exercised against it. Hence, if heliozoan locomotion does provide the force for division, it is only in those circumstances where the organism is attached to some solid object.

(c) Microtubules as force transmitters

Cold and colchicine destroy axonemal microtubules in heliozoa (Tilney and Porter, 1967; Tilney, 1968), and completely inhibit the separation of daughter Actinophrys. It is likely, therefore, that division depends upon the presence of microtubules. Microtubules are strong, elastic structures (Chapter IV), so their essential role probably lies in the transmission of some force required to separate the daughter organisms. In free-floating dividers this must be a "pushing" force. To function effectively, such a force must operate in the region between the two dividing cells. The requirement then is probably for a microtubular force-transmitting component which lies in the region between the two dividing daughter Actinophrys.

(d) Axopodia as force transmitters

The fine structural evidence rules out the possibility that these microtubules are contained in the bridge of cytoplasm between the cells. The finding that vacuoles occlude the cytoplasmic bridge indicates the same conclusion.

The only microtubule containing components which span the space between the cell bodies of floating daughter organisms are the axonemes of those axopodia, based on opposite cell bodies, which contact each other. Evidence will be presented in Chapter IV which reveals that axopodia elastically resist bending; a bent axopodium therefore is normally an indication of a deforming force. Some of the axopodia which do make contact between dividing cells are also bent (Plate 24). They may be

bent because they are transmitting the force which is separating the daughter organisms.

(e) Alternative modes of division

It is possible that, in all cases, the force separating daughter Actinophrys is transmitted by axopodia. In the case of organisms attached to the bases of Petri dishes this is probably a "pulling force" exerted against the bases of the Petri dishes. According to Watters (1968), leading axopodia shorten when their tips are firmly attached to the substrate and thus cause daughter organisms to move about. It has been argued above that the axopodia of free-floating dividers must exert a "pushing" force against other axopodia.

These division processes may not be distinctly separate. They could represent extremes of a continuous range in which organisms undertake intermediate forms of division when some axopodia "pull" on the substrate and some axopodia "push" against the substrate and/or axopodia on the other daughter Actinophrys. Such an arrangement could explain the variation in rates of division shown in Table IV (above, p. 26). The chances of an axopodium from a floating organism making contact with another axopodium would seem to be far lower than the chances of an axopodium from a substrate-attached organism making contact with the bottom of the Petri dish. The free-floating division could be thought of as a more "difficult" type of division which takes the Actinophrys longer to accomplish.

(f) Axopodial growth

How are the contacts made between "pushing" axopodia on free-floating dividers? Newly forming axopodia are situated in the area where such contacts occur. The continual growth and resorption of these axopodia may represent a trial and error system for forming contacts. When a

correct contact is made by an axopodium it might "trigger" continued growth where otherwise the axopodium would be resorbed.

(g) Force production

A complete discussion of possible mechanisms of force production in division would include a review of the several theoretical models which have been proposed by cell biologists; most of these deal with the mechanics of the spindle. Models of two main types can be considered. One type suggests that force production occurs by addition or loss of oriented molecules (e.g. Inoué and Sato, 1967), and the other attempts to explain movement in terms of a "sliding or shearing" mechanism (e.g. McIntosh, Hepler and Van Wie, 1969). There are recent reports of actin localised in the spindles of locust (Gawadi, 1971) and crane fly (Forer and Behnke, 1972) spermatocytes. As Huxley (1973) has shown there are great similarities at the ultrastructural and biochemical levels between many apparently unrelated types of force-producing mechanisms. All of the systems he has reviewed appear to contain oriented actin filaments.

In conclusion, it appears that the force required to divide Actinophrys is probably transmitted by axopodia; it is not known if axopodia are involved directly in the production of this force.

(h) Similarity to Amoeba

The theory of division presented here for Actinophrys is similar in all but matters of terminology to the model proposed by Chalkley (1935) solely for Amoeba proteus. The most remarkable agreement is in the case of free-floating division, which also occurs in Amoeba (Chalkley, 1935). Both organisms appear to require a modified fission process to undertake free-floating division. In Actinophrys this seemingly involves axopodia "pushing" on axopodia whereas in Amoeba the cell becomes stellate and the unusually long and thin pseudopodia apparently "push" against each other.

It is clear from the work presented here that this method of division may well have a wider application. The class Rhizopoda contains the organisms most likely to use this system, but any cells which have a discrete nuclear and cytoplasmic division and which do not round up and become immobile during fission may have the potential to divide in this way.

CHAPTER III

REPOSITIONING OF AXONEMES AND THEIR MICROTUBULES

Introduction

Results presented in this chapter will describe changes in the position of axonemes, or changes in relative position of microtubules within axonemes. A discussion of these results will consider the possibility that axonemal components require to achieve a stable minimum-energy state, the fully organized axoneme.

At a stage before nuclear division, axonemes become detached from the nuclear envelope (Belar, 1923). A study of the means by which axonemes reattach to the nuclear envelope, just before binary fission, indicates that microtubules are repacked into the normal axonemal pattern, as a distinct process, after they have been polymerised.

A polarised repair process, of bends in axonemes, appears to depend on tubule repacking. Exploiting these bends as "markers", information has been gained about the mechanism of action of the alkaloid colchicine on microtubules.

In Actinophrys, whole axonemes appear to change their relative position. Fusion of neighbouring axopodia has been studied. Such fusions apparently entail the movement together of previously separated axonemes.

These observations in Actinophrys are comparable to those of Tucker (1970). He has shown that in the ciliate Nassula large bundles of microtubules, formed at one point in a cell, move and become reattached at another site where they become functional. Such deployment processes extend present concepts of the role of microtubules in morphogenesis. It

has been suggested that microtubules are one state in a reversible equilibrium reaction between oriented and non-oriented molecules (Marsland, Tilney and Hirshfield, 1971). This equilibrium has been analysed in the same thermodynamic terms that Inoué (1964) used to analyse birefringence changes occurring in spindle fibres.

This idea, of tubules and subunits being in a reversible equilibrium, is a very valuable insight into the microtubule self-assembly process; however, it has unfortunately led to an oversimplified view of the role of microtubules. For example, it is often assumed that microtubules exist and function where they are made, attached to some fixed "nucleating site". When there is no further requirement for the tubules at that position, it is assumed, conditions alter in some way, thus causing the tubules to break down again into their subunits.

Such a "static" view of microtubules should be extended to include the dynamic events reported in this chapter, where it appears that whole microtubules move about the cell, rather than first depolymerising into subunits and then repolymerising at another "nucleating site".

Results

(a) Axonemes detached from the nuclear envelope

Actinophrys from a 4-day-old culture are fixed and embedded (Materials and Methods). An examination of this embedded material reveals that a distinction can be made between early and late dividers on the basis of cell shape. In early dividers axonemes apparently do not make contact with the nucleus. Sequences of sections show that this appearance is not caused by the axonemes passing out of the plane of the section. The lowest of the 3 axonemes in Plate 34 remains in the plane of the section until it enters an axopodium (this represents a length of 20μm). On the

surface of the nuclear envelope and in the cytoplasm adjacent to it small densely staining granules (approx. 600 μm diameter) are apparent (Plate 34).

(b) Reattachment of axonemes to the nuclear envelope

Late dividers contain axonemes of unusual morphology which contact the nuclear envelope (Plate 33). It is likely that these axonemes exhibit stages in reattachment to the nuclear envelope.

The bases of the reattaching axonemes are "splayed". They are incomplete because they often contain fewer microtubules than are visible in the distal region where the usual axonemal pattern remains. Short sections of microtubules have orientations which are not radial. Certain microtubules extend from the base of the region of the axoneme, where the tubules are packed in the usual fashion, to parts of the nuclear envelope not directly below the axoneme.

(c) Bending and shearing of axopodia

The axopodia of organisms, sucked up and down in a clear glass pipette, tend to stick to the glass as they brush against the pipette wall. This process damages axopodia. Some become very much shorter because the distal portions of axopodia are lost. This is probably because they are sheared off in the pipette. During the 20 min period after damage, the average rate of outgrowth of 3 axopodia, shortened in this way, each on a separate organism, is 2 μm min^{-1}. Initially these axopodia are in the range 25-55 μm long. During the first 5 min of the recovery period the rate of outgrowth is higher than the average rate (4 μm min^{-1}).

Other axopodia are bent elastically into even curves. Axopodia bent in this manner frequently adhere to other axopodia or the glass slide at their tips. Eventually the adhesion fails and these curved axopodia "spring" back to the straight form in one sudden movement.

Some of the axopodia yield non-elastically and a bend localised at some point along the longitudinal axis is formed. If the axis is bent through an angle greater than about 70° then the distal portion of the axopodium swings round and fuses with the portion of the axopodium proximal to the bend. The subsequent behaviour of axopodia affected in this way was not studied.

(d) The polarised repair process

A repair process restores the normal form to axopodia which contain bends of less than about 70°. In this repair process the bend moves distally out along the axopodia. It accomplishes this at a fairly uniform rate in the range 10-14 μm min^{-1} (Fig. 11). Frequently the angle of the bend becomes less acute as it moves out along the axopodium. The total length of the axopodium does not change significantly during the repair process. Haptocyst movement continues in the region of the axopodium distal to the bend. Haptocysts also move past bends apparently unhindered by them in any way.

(e) Colchicine and the repair process

If organisms are immersed in 0.5% colchicine their axopodia begin to shorten at rates of up to 50 μm min^{-1} within 1 or 2 min (Fig. 12; Plates 88, 89). When organisms with bent axopodia are placed in colchicine of this concentration the distal movement of a bend along an axopodium still occurs at the normal rate even while that axopodium is shortening (Fig. 12). The rate of shortening of bent axopodia after colchicine treatment is approximately the same as the rate of shortening for normal axopodia treated in the same way.

(f) Fusion of axopodia

Occasionally, after damage of the type just described, after low

FIGURE 11

Shows changes in the distance between a bend in the longitudinal axis of an axopodium and the base of the axopodium (open circles). The base to tip length of the axopodium is also shown (black circles).

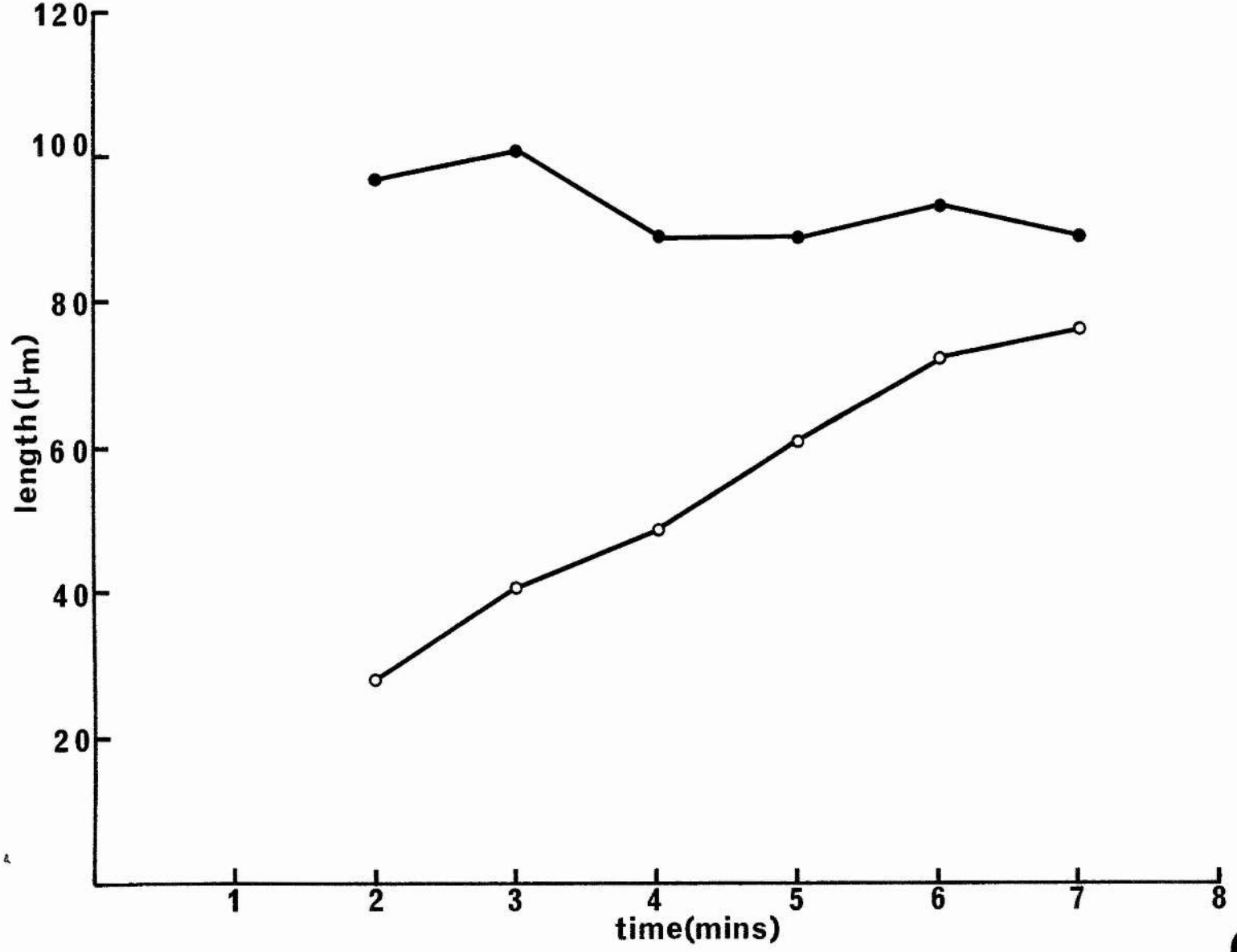
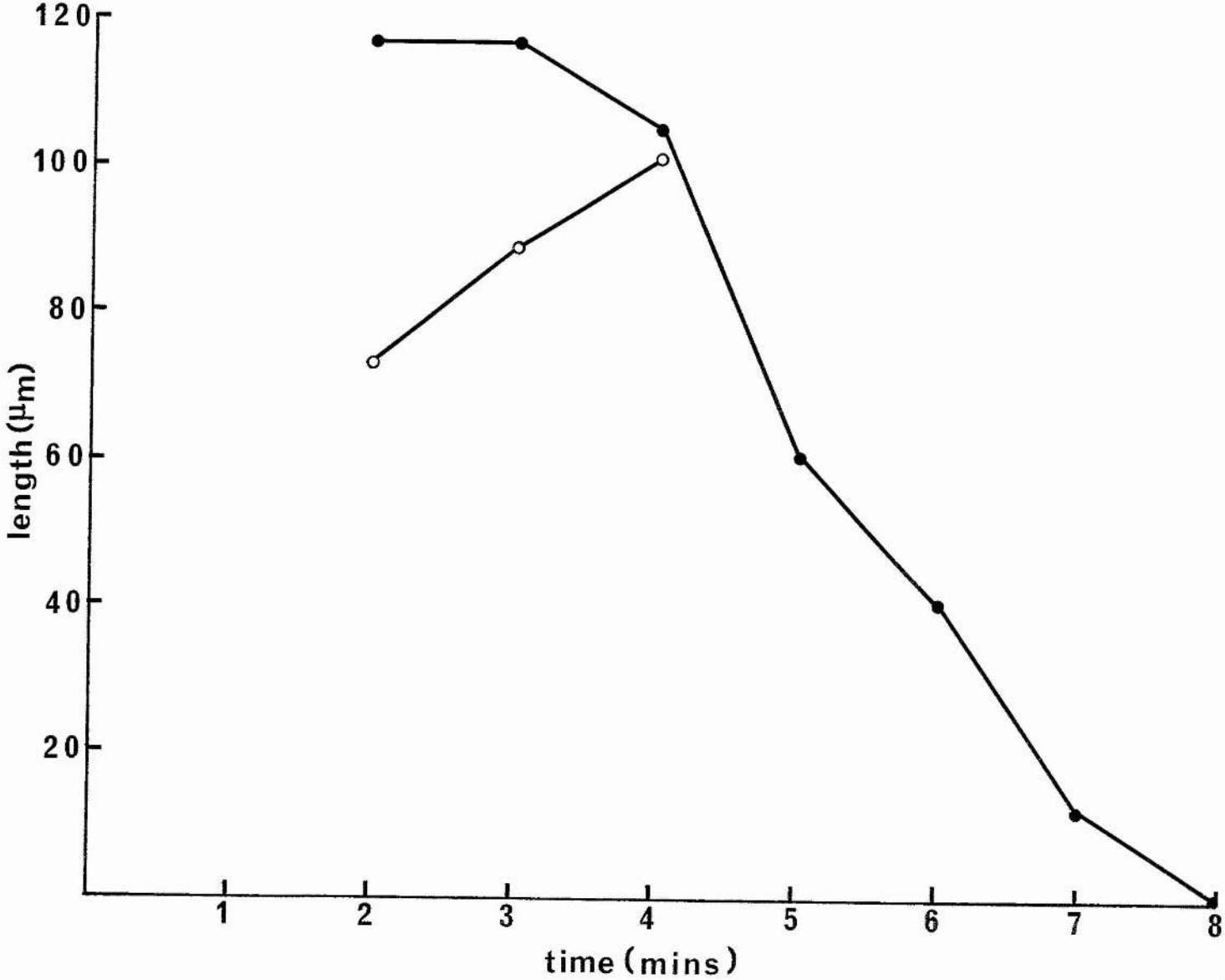

120
100
80
60
40
20
length(μm)
1
2
3
4
5
6
7
8
time(mins)

FIGURE 12

Shows changes in the base to tip length of an axopodium (black circles) shortening in an organism immersed in a solution of colchicine. Changes in the distance between a bend in the longitudinal axis of this axopodium and its base (open circles) are also shown.


120
100
80
60
40
20
length (μm)
1
2
3
4
5
6
7
8
time (mins)

temperature treatment, or during regrowth of axonemes after rapid contraction (Chapter V), two neighbouring axopodia fuse along their entire lengths. This fusion starts at the base and proceeds gradually towards axopodial tips (Plates 35-37). It progresses at a roughly constant rate of about $10\mu m\ min^{-1}$.

A few examples of axopodia containing two axonemes are observed (Plate 38). These may be the result of axopodial fusion. (Echinosphaerium also appears to possess axopodia with two axonemes (Kitching and Craggs, 1965; Tilney and Porter, 1965).)

Discussion

(a) Localised breakdown of part of an axoneme

Bases of axonemes which are detached from the nuclear envelope are not flat or "stepped" as they are in interfission organisms. They have an irregular shape because certain tubules extend down towards the nucleus further than others. This indicates that microtubule tips at the bases of axonemes have been broken down. The localised breakdown of microtubules can be extremely precise (Tucker, 1971; Williams and Frankel, 1973). In this case the outstanding feature is that only a part of the organelle, the very base, is degraded. This breakdown may have adaptive significance for two reasons. It may facilitate the even distribution of parental axonemes over the surface of the two newly formed nuclei. It may also be that such very long axonemes would be a mechanical hindrance to nuclear division if they remained attached to the nuclear envelope. Belar (1923) has shown that nuclear division in Actinophrys apparently occurs without breakdown of the nuclear envelope.

(b) Reattachment of axonemes

The form of the axoneme "reattaching" to the nuclear envelope

demands careful consideration. It may have arisen by one of two processes, either by the formation of new microtubules or by the sliding downwards of already polymerised microtubules from the completely formed region of the axoneme. The fact that the packing of microtubules in the axoneme immediately above this region appears entirely normal does not eliminate the second alternative because microtubules may be continuous throughout the length of the axoneme. The presence of a large number of polyribosomes in the region of the "splayed" microtubules suggests that the synthesis of some protein is occurring.

There is a correlation between the number of ribosomes in a polysome and the molecular weight of the protein it is synthesising. The molecular weight of mammalian brain tubulin is 57,000 (Fine, 1971). Assuming a similar molecular weight for heliozoan axonemal tubulin, the number of ribosomes expected in a polyribosome coding for it can be deduced. Reference to Sommerville's (1967) calibration graph gives an approximate value of 13. It may not be without importance to notice that 13 is also one of the more commonly quoted numbers of subfilaments which are said to make up the wall of a microtubule (Porter, 1966).

Because of the length of such polyribosomes (about 117 nm) they will be seen as whole structures infrequently in electron micrographs of sectioned material. Polysomes in this situation in micrographs of Actinophrys contain at least 5 ribosomes (Plate 34).

The evidence would suggest then that at least some protein required for the formation of new axonemes is being newly synthesised. The evidence that this is tubulin is weak. It seems unlikely that already constructed microtubules slide down out of the axoneme into this region, but no direct evidence can be presented against it. Synthesis and polymerisation of tubules from a pool of already synthesised precursors cannot be excluded either.

The microtubules in this region are probably growing. This is

apparently the first reported electron microscope observation of growing microtubules in any heliozoan. All other "growing axopodia" have in fact been reaggregating after experimental degradation (Roth and Shigan-aka, 1970).

(c) Repacking of microtubules in the reattaching axoneme

It is clear from the morphology of such axonemes that in order to achieve the microtubular configuration typical of a normal axoneme, either microtubules will have to move towards each other or those tubules which are wrongly placed will have to sequentially disaggregate then grow out again and again in new directions each time until the correct direction is taken. I do not favour this latter suggestion. It would require individual microtubules to be "recognised" by particular sites.

An alternative theory suggests that tubules are repacked. One way to envisage this is that tubules near the correctly packed region are vibrating as a result of Brownian movement or in some other way. When they are at the correct spacing a cross-link would become inserted and the structure would be stabilized. In this way the microtubules of the axoneme would become more ordered as a greater number of cross-links became inserted.

(d) Repair of bent axopodia

Observations indicating the presence of a moving repair zone are important, because of their relevance to microtubular packing in the axoneme, and because they suggest axonemal polarity.

In the first place it is clear that bends always migrate distally, never centripetally. This requires that there is a definite polarity in the axoneme. The physical basis of this polarity is not known, but could lie in the heterodimer subunit proposed by Bryan and Wilson (1971).

The nature of the damage that is being repaired is of great interest.

Cross-linked microtubules in the axopodium provide structural support (Tilney and Porter, 1967). Therefore bends of this kind in axopodia must be caused either by total breakage of microtubules or by local disruption of cross-links and bending of microtubules. Whereas total breakage of microtubules may have occurred in those bends in axopodia greater than 70^{o} it seems less likely to have occurred in the case of those bends which undergo a polarized repair process. The bends move outwards at rates ($12 \mu m\ min^{-1}$) which are faster than the rate of outgrowth of undamaged axopodia of the same length ($6 \mu m\ min^{-1}$). It is probable therefore that there is no complete break and that the microtubules are just bent and disarranged in some fashion.

(e) Mechanism of action of colchicine

Tilney (1968) has shown that microtubules in heliozoan axopodia are disaggregated by colchicine. Figure 12 shows that the effect of colchicine on bent axopodia is always to shorten the section of the axopodium which is distal to the bend. Therefore it can be concluded that microtubules in the heliozoan axopodium are broken down, with this concentration of colchicine, by action at their tips.

The possibility that microtubule subunits are constantly being freed from and being incorporated into microtubules has already been considered. In view of this and the evidence presented above it seems possible that colchicine could be acting by attaching to the subunits as or after they dissociate from the microtubule. The resulting subunit-colchicine complex it is supposed would not be able to re-enter a microtubule. Thus a progressive shortening of microtubules from their tips would occur.

(f) The axoneme — a minimum energy state?

When the components of the axoneme are highly ordered it is presumed

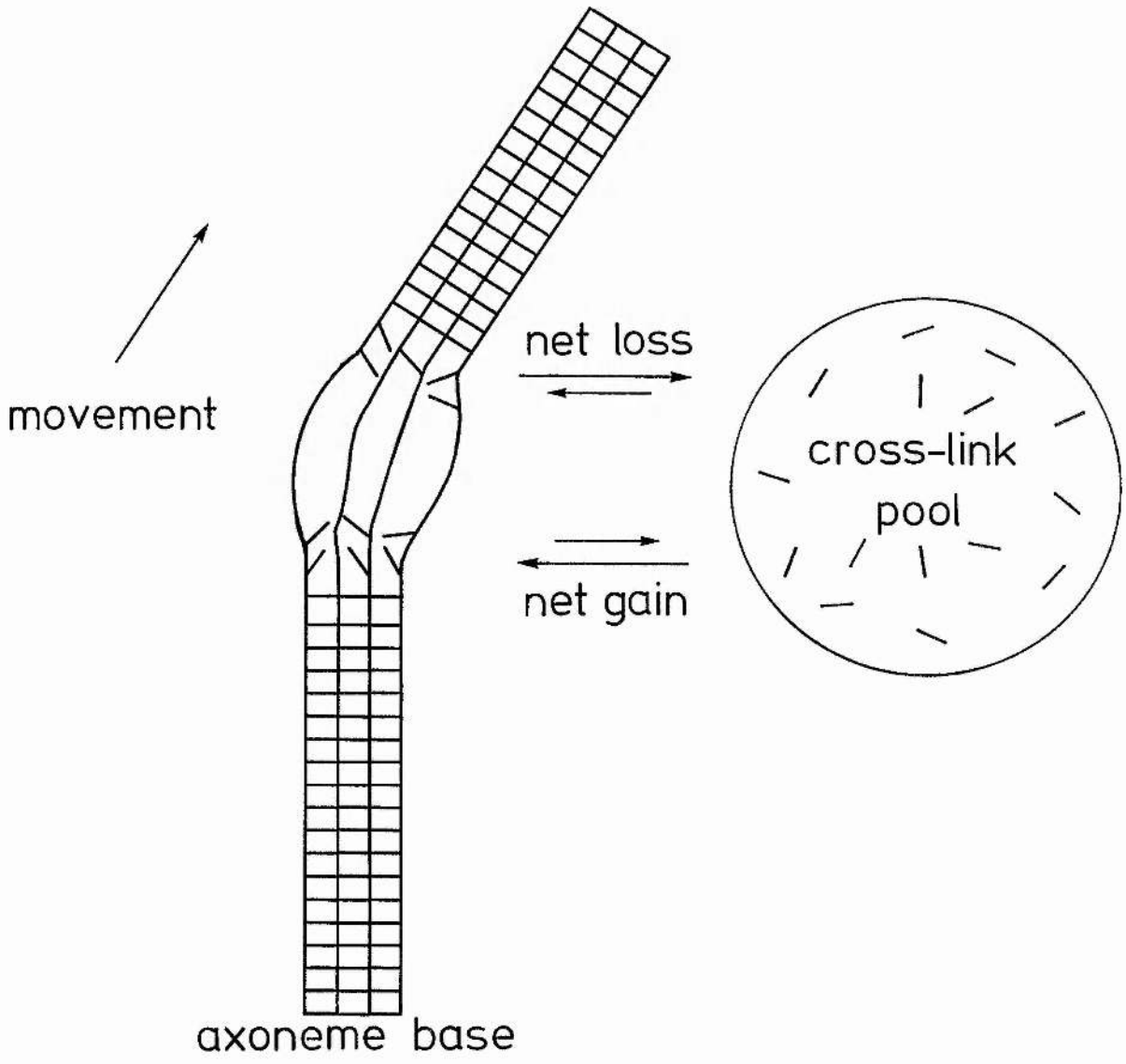
that a maximum number of binding sites on microtubules and cross-links are occupied. Damage, in the form of permanent bends in the axopodia, can be viewed as an input of energy to the system. Many cross-links might now be highly reactive because they have less than a satisfactory number of binding sites for microtubules occupied. Others are presumably holding to incorrect sites thus bending microtubules which are tending to return to the straight form. It is possible that an "auto-repair process" takes place making use of this "stored" energy. Microtubules and cross-links could move and vibrate as a result of Brownian motion until chance encounter of suitable linking sites causes a "correct" bond to be formed. On contact the energy of the unsatisfied binding site would be released into the bond, thus stabilizing the pattern of the axoneme. Those cross-links in "incorrect" positions would have their bonds strained by bent microtubules and so would tend to break more frequently than those in "correct" positions, and thus through reattachment of links to correct sites "incorrectly" placed cross-links would diminish in number.

As a description of a possible course of events at the molecular level this is adequate as an explanation of bend repair only up to a point. It does not explain why the repair zone moves.

If, as has been suggested for microtubule subunits, cross-linking material (nexin?) is also in an equilibrium between the bonded and unbonded form then cross-links could be being made and broken continually at both ends of the repair zone. If for some reason bonds are made more frequently or break less often in the part of the repair zone nearest the cell body than in the part furthest from it a situation could arise where the former region was becoming more stable at the expense of the latter via an intervening pool of free cross-linking material (Fig. 13). Long-range allosteric effects as discussed by Roth et al. (1970) would be one basis on which such a difference in affinity for cross-links could be

FIGURE 13

Illustrates an interpretation of the way in which bands may migrate distally along axopodia.


movement
net loss
net gain
cross-link
pool
axoneme base

founded.

Schemes of this type are of course very useful for visualising events which take place at this level of organization. The evidence however that organelles as complex as this can self-assemble is virtually non-existent. Experiments *in vitro* of the type which analyse the self-assembly of T.M.V. (Durham, Finch and Klug, 1971), bacterial flagellae (Asakura, Eguchi and Imo, 1966) and individual microtubules (Weisenberg, 1972) from their subunits must be performed with the isolated constituents of whole axonemes. If these give rise to recognisable patterns similar to those seen in the heliozoan axonemes *in vivo*, then a much firmer basis for interpretations of the type offered above will have been established.

CHAPTER IV

THE ELASTIC PROPERTIES OF AXOPODIA

Introduction

Although many authors agree that microtubules are cytoskeletal in function (Tilney and Porter, 1965; Brinkley and Cartwright, 1971) this agreement is based on simple correlations. When long, thin cell extensions develop, microtubules are commonly found within them oriented in the direction of their longitudinal axes (reviewed by Porter, 1966). Conversely, when agents which cause microtubules to disaggregate are applied, the long cell extensions are usually lost (Tilney, 1969; Roth and Shigenaka, 1970).

Axopodia are cell extensions of this type; they contain a large number of compactly arranged microtubules along their entire lengths. An investigation of the elastic properties of the axopodia of Actinophrys should hence provide more direct evidence in support of the widely held belief that tubules perform a cytoskeletal role.

Results

(a) Micromanipulation of axopodia

Axopodia frequently adhere to glass microneedles which brush against them. If this brushing movement is gentle, and the attachment is made near the tips of axopodia, they are elastically bent into an evenly curved shape (Plate 42). The axopodia eventually detach from the needles. When this occurs they spring back, vibrate about their original straight

position for a fraction of a second, and then come to rest.

More powerful manipulations of the glass needle often produce more severe bending of axopodia; the axopodia yield non-elastically (Plate 44). These bends are similar to some of the bends formed by shearing in fine glass pipettes. Axopodia damaged in this way do not spring back to their original straight form when released.

(b) Estimation of Young's Modulus for an axopodium

A glass fibre of approximately the same diameter as an axopodium was mounted on a micromanipulator. The tip of the glass fibre was drawn into contact with the very tip of an axopodium. It became firmly stuck to the axopodium, and was then moved further in the same direction causing bends to appear in both the axopodium and the glass fibre. This configuration was photographed (Plate 43) and the negative was made into a transparency for projection. In this equilibrium situation there is a relationship between (1) the strengths of the two components (the axopodium and the glass fibre), (2) their diameters, and (3) the radii of curvature of the two bends. This relationship is expressed in the following equation:

$$E_1 = E_2 \cdot \frac{R_1}{R_2} \left(\frac{a_2}{a_1}\right)^4 \quad . \quad . \quad . \quad . \quad (1)$$

where E_1 = Young's Modulus for the axopodium,

E_2 = " " " " glass fibre,

a_1 = radius of the axopodium,

a_2 = " " " glass fibre,

R_1 = radius of curvature of the bend in the axopodium,

R_2 = " " " " " " " " glass fibre.

The derivation of this equation is contained in Appendix I.

(i) Young's Modulus for the glass fibre (E_2)

Young's Modulus can be assessed for a material using a sample of the material of any shape or size (Weidner and Sells, 1968). The glass fibre used in this experiment was prepared by drawing out the tip of a glass microelectrode-tube on a microforge. Determination of Young's Modulus for the glass was made using one of these tubes, not the fibre itself. The measurements which are required, and the calculation of the modulus, are included in Appendix I.

Minimum $E_2 = 3 \times 10^{11}$ dynes/cm^2

Average $E_2 = 4.8 \times 10^{11}$ " "

Maximum $E_2 = 6.6 \times 10^{11}$ " "

(ii) Radius of the axopodium (a_1)

The transparency of the axopodium was projected onto a large sheet of graph paper and the diameter of the axopodium was measured at 10 different points along its length. Because the outline of the axopodium was not perfectly sharp at this magnification, maximum and minimum values were assessed at each of the 10 points. An average diameter and radius were calculated using the maximum and minimum sets of values.

minimum diameter	maximum diameter
2.1	4.8
1.5	5.0
1.6	5.3
2.5	5.2
1.7	5.1
2.1	3.6
2.4	5.7
2.3	5.2
2.2	5.2
2.2	5.9
10 | 20.6	10 | 51.0

AV. DIAMETER (min) 2.06 AV. DIAM. (max) 5.1

Hence, minimum possible a_1 = 1.03 units,

and maximum " " = 2.55 "

(iii) Radius of the glass fibre (a_2)

The diameter of the glass fibre was also measured from the projected slide. An average diameter was calculated from measurements made at 8 points along its length. Maximum and minimum possible measurements were made at each point in the same way as for the axopodium.

minimum diameter	maximum diameter
2.4	5.0
2.6	6.3
2.3	4.0
1.7	5.7
2.5	5.7
2.4	6.2
2.2	4.5
1.7	3.6
8 | 17.8	8 | 41.0
AV. DIAMETER (min) 2.2	AV. DIAM. (max) 5.1

Hence, minimum possible a_2 = 1.1 units,

and maximum " " = 2.55 "

Radii were assessed in arbitrary units. Because these appear as a ratio in Equation (1) this does not affect the calculation. The real average diameters for both the axopodium and the glass fibre were approximately:

maximum possible diameter 3 μ_m

minimum possible diameter 1.5 μ_m

Similarly the radius of curvature of the bent axopodium (R_1) and the bent glass fibre (R_2) can be measured in arbitrary units.

(iv) Radius of curvature of bend in axopodium (R_1)

The tip of the axopodium (T) moved a perpendicular distance (e_1) from the original axis of the axopodium (Fig. 14). The base to tip length of the axopodium is (d_1).

Now because e_1 is small compared with R_1

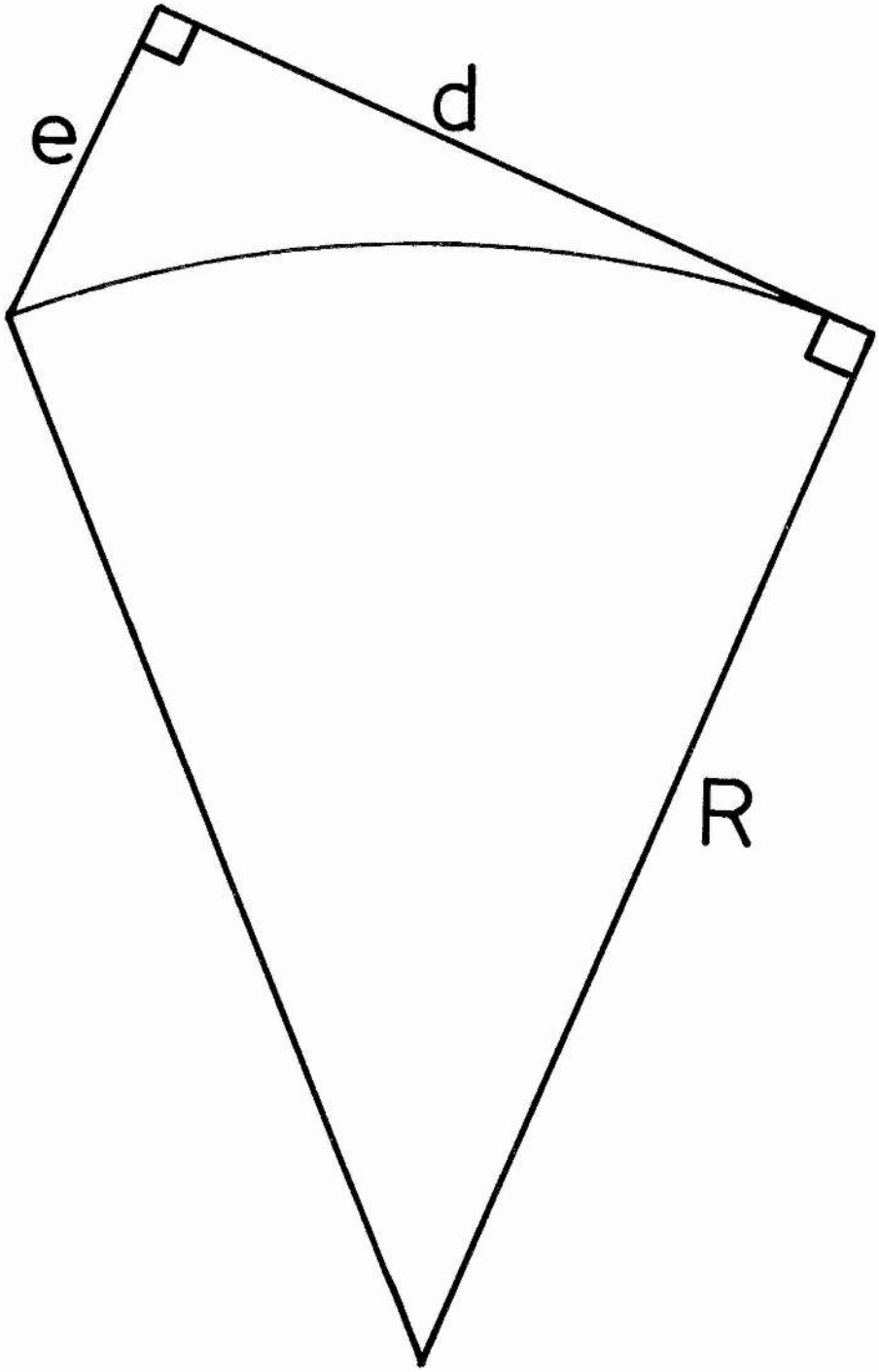
$$R_1 = \frac{d_1^2}{2e_1} \quad . \quad . \quad . \quad . \quad . \quad (2)$$

FIGURE 14

An illustration of a gently curved line (a bend therefore with a large radius of curvature — R). In instances, like those under consideration, where the deflection e of the axopodium and glass fibre from the vertical is small and their radius of curvature is large the formula

$$R = \frac{d^2}{2e}$$

is applicable.



when $R \gg e$

$$R = \frac{d^2}{2e}$$

The lengths d_1 and e_1 were measured on a large sheet of paper onto which the magnified profiles of the axopodium and glass fibre had been drawn (Fig. 15) by following the outlines when the transparency was projected onto the paper. Errors made in these measurements are negligible compared with those made in assessing a_1 and a_2.

Substituting in Equation (2),

$$R_1 = \frac{218^2}{20.6}$$

$$= 1153.5 \text{ units}$$

(v) Radius of curvature of bend in glass fibre (R_2)

To assess the radius of curvature of the bend in the glass filament the paper tracing was used again. A tangent to the curve was drawn at an arbitrary point (Q). The tangent was constructed by placing a mirror on the curve with the reflecting surface at right angles to the plane of the paper. The mirror was rotated until the tracing of the bend and its reflection formed an even curve. At this point, the plane of the mirror was at right angles to the tangent of the curve. The line (AB), representing the reflecting surface of the mirror, was drawn onto the paper. Next a perpendicular was dropped onto AB at the point Q where it intersected with the curve. This perpendicular was a tangent to the curve at the point Q (Fig. 15).

Values for d_2 and e_2 were then measured. Although the error in d_2 is negligible, small inaccuracy in construction of the tangent QP leads to a potential error in the measurement of e_2. For this reason highest and lowest possible values for this length are given. These were substituted in the equation

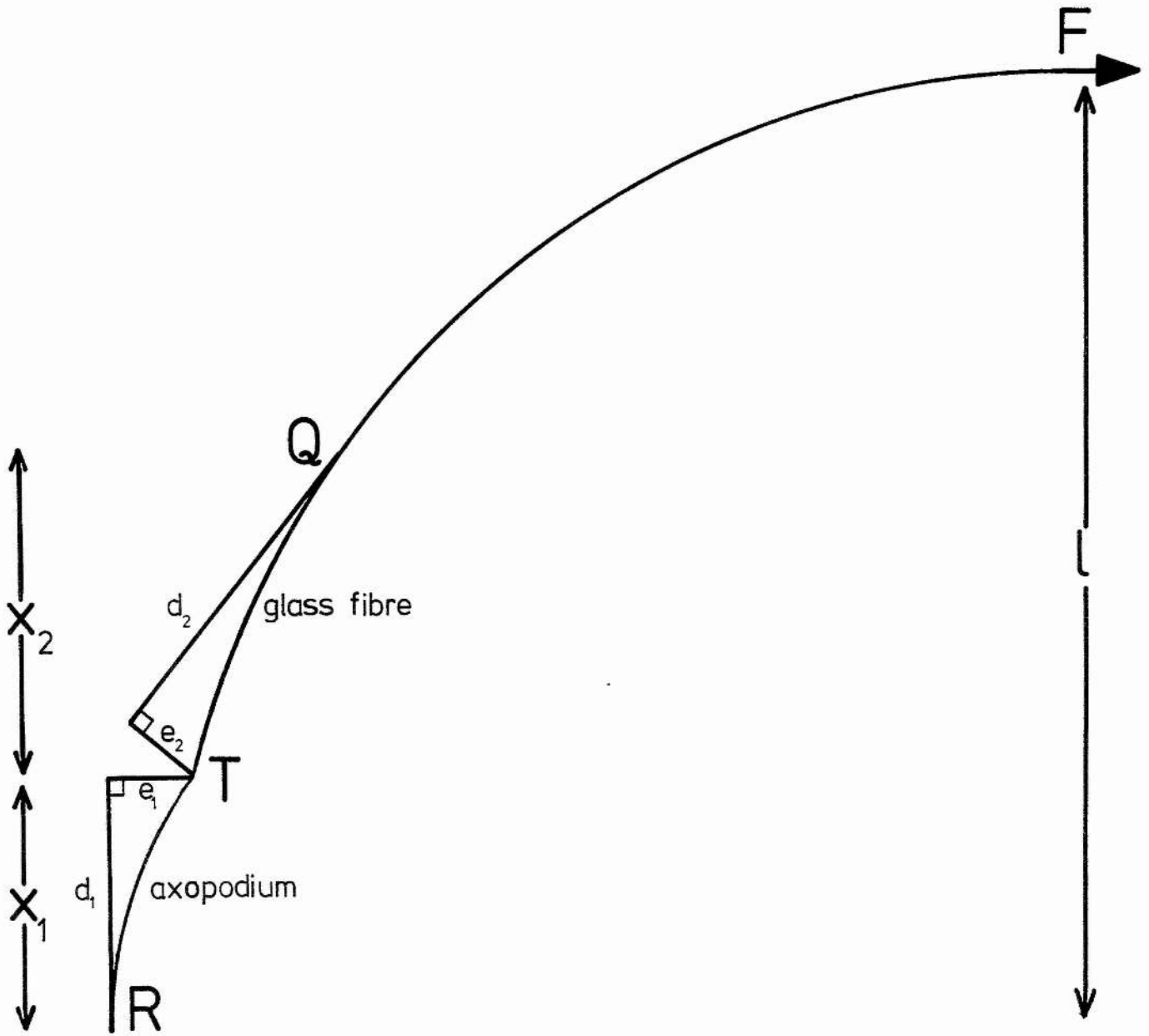
$$R_2 = \frac{d_2^2}{2e_2} \quad . \quad . \quad . \quad . \quad . \quad (3)$$

Using the highest possible value for e_2,

$$\text{minimum possible } R_2 = \frac{133.5^2}{29.4} = 606.2 \text{ units.}$$

FIGURE 15

A diagram illustrating the equilibrium situation in which both the glass fibre and the axopodium shown in Plate 43 are bent.


F
Q
L
X_2
d_2
glass fibre
e_2
T
e_1
X_1
d_1
axopodium
R

Using the lowest possible value for e_2,

$$\text{maximum possible } R_2 = \frac{133.5^2}{24.6} = 724.5 \text{ units.}$$

(vi) Young's Modulus for the axopodium (E_1)

Enough information is now available for the calculation of maximum and minimum possible values of E_1 for the axopodium. Substituting values determined above in Equation (1) (the reasoning underlying the selection of values of a_1 and a_2 for substitution is explained in §(c) of the Discussion),

$$E_1 = E_2 \cdot \frac{R_1}{R_2} \left(\frac{a_2}{a_1}\right)^4 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

$$E_1 \text{ maximum} = 6.6 \times 10^{11} \times \frac{1153.5}{606.2} \times \left(\frac{1.1}{1.03}\right)^4$$

$$= 1 \times 10^{12} \text{ dyne/cm}^2$$

$$E_1 \text{ minimum} = 3 \times 10^{11} \times \frac{1153.5}{724.5} \times \left(\frac{2.55}{2.55}\right)^4$$

$$= 5 \times 10^{11} \text{ dyne/cm}^2$$

Young's Modulus for this axopodium lies in the range

$$5 \times 10^{11} - 1 \times 10^{12} \text{ dyne/cm}^2.$$

Discussion

(a) Cytoskeletal role of the axoneme

Axopodia are extremely long and thin (diameter $\simeq \frac{\text{length}}{200}$), yet they elastically resist bending. Both the axoneme and the cell membrane extend the full length of axopodia. Thus it seems that structural support must be provided by one or other of these components.

To distinguish between the two alternatives it is necessary to examine more closely the observation that when bends greater than 70° are made in axopodia the distal portions of axopodia swivel and fuse with the proximal portions. At the time when axopodia swivel, the external cell

membrane is still intact. It seems likely that the cell membrane alone cannot support the distal portion of the axopodium. Therefore it is probable that the axoneme is the structure mainly responsible for the resistance to bending. In this situation it is possible to show, more directly than in any other, that microtubules have certain properties which fit them for their suggested cytoskeletal role. They are stiff and elastic.

The structure of the axoneme is consistent with the suggested function. It is a fact, widely appreciated amongst engineers, that a tube of a particular radius and material is both lighter and stiffer than a rod of the same radius and material (Alexander, 1971). The form of microtubules therefore represents an excellent shape for a skeletal unit of this type. The arrangement of microtubules in the axoneme has been discussed by Kitching (1964). He concluded that the "double spiral arrangement", although not quite as effective as an arrangement of microtubules in concentric circles, is nevertheless one of the patterns most suited to resisting flexure and torsion.

(b) The strength of materials — a comparative analysis

Table VI contains values of Young's Modulus for various materials. Those of animal origin fall into two distinct classes. The first behave like soft rubbers which have a high resilience and a value for Young's Modulus of about 10^7 dyne/cm^2. The second are more rigid. These have a Young's Modulus greater than 10^9 dyne/cm^2 and are skeletal materials of accepted importance as force transmitters. The value of E_1 for the axoneme falls into the second class. Thus the axoneme is as stiff as the skeletal materials which occur in more bulky forms in larger multicellular animals and hence microtubules appear to be well fitted to their suggested cytoskeletal function. Microtubules may be the strongest organic structural supporting material in the animal kingdom.

TABLE VI: Young's Modulus for various materials

Material	Young's Modulus ($dyne/cm^2$)	Reference
Elastin	6×10^6	Bergel, 1961
Abductin	1.4×10^7	Kelly and Rice, 1967
Resilin	1.8×10^7	Weis-Fogh, 1961a
Collagen	10^{10}	Harkness, 1961
Bone	10^{11}	Smith and Walmsley, 1959
Insect cuticle	10^{11}	Jensen and Weis-Fogh, 1962
Ivory	1.72×10^{11}	Gordon, 1968
Cross-linked microtubules	5×10^{11}-1×10^{12}	My value
Lightly vulcanized rubber	7×10^6	Ferry, 1970
Oak	10^{11}	Hodgman, 1965
Glass	$3\text{-}7 \times 10^{11}$	My value
Mild steel	2×10^{12}	Hodgman, 1965

Harris (1961) calculated that the couple required to bend a cilium into an arc of radius $6\mu m$ was 5×10^{-10} dyne/cm. He made the assumption that the central pair of microtubules alone were responsibe for maintaining the stiffness of the cilium. This enabled him to suggest a value of 3×10^{11} $dyne/cm^2$ for the Young's Modulus of the central pair of microtubules. Allowing for the fact that some stiffness is probably supplied by all "9 + 2" ciliary microtubules, and for the fact that because of their function they are less likely to be as firmly cross-linked as the microtubules in the heliozoan axoneme, Harris's value is reasonably compatible with my experimentally determined value. In his discussion of this aspect of Harris's (1961) work, Sleigh (1962) states that because the Young's Modulus of biological fibres is unlikely to exceed 10^9 $dyne/cm^2$ the rigidity of the cilium is probably not supplied by ciliary microtubules. He supports an alternative theory of Harris's, which assumes that ciliary

resistance to bending is supplied by internal turgor pressure. Referring to Table VI, it is clear that Sleigh is not entitled to reject the first theory on the grounds that biological fibres are unlikely to have sufficient ($E = 10^9$ dynes/cm^2) resistance to bending.

(c) Assumptions and accuracy of the calculation of E_1

A point which must be emphasised is that this experiment was performed only once. It exploited available techniques to the limit and was made possible because, luckily, a photograph of an axopodium and glass fibre bending against each other was obtained. Many other attempts to repeat the experiment were unsuccessful. In view of the importance of obtaining some direct measurement of the stiffness of microtubules even a single assessment is worth recording.

At all stages in the calculation, the errors introduced during measurement were taken into account, by using a range of possible values rather than an average value. The most inaccurate measurements were those of the diameters of the extremely thin axopodium and glass fibre. The ratio of these diameters is expressed to the 4th power in Equation (1). In this situation a comparison of the highest possible value of a_1 with the lowest possible value of a_2 and vice versa would have resulted in a meaninglessly large range of values for E_1. Since a_1 and a_2 were measured in the same way it was assumed that excessively large assessments of one would be accompanied by proportionately large assessments for the other. Similarly if the assessment of one was excessively low it was assumed that assessment of the other would also be proportionately low. Hence where the maximum value of a_1 was used in Equation (1) the maximum value of a_2 was also used. The minimum values were used together in the same way. In fact it seems quite reasonable that if for some reason the image of the axopodium is too broad (e.g. as a result of poor focus) then the image of the glass fibre will also be too broad.

The validity of Equation (1) as a description of the axopodium/glass fibre situation depends upon the appropriateness of certain assumptions. Equation (1) has been formulated for the bending of narrow beams. The beams (axopodium and glass fibre) must be thin. This requirement is adequately satisfied because they are about $2^{\mu}m$ thick. As the measurements show, the degrees of tapering of the axopodium and glass fibre in the bent regions are negligible. The axopodium and glass fibre may be considered as having a uniform cross-section, which is a further requirement of the equation. In order to satisfy the equation, Young's Modulus should be uniform across the cross-section of the beams. The constant spacing of tubules in axonemes suggests that this is likely to be the case; the axopodium and the glass will presumably have a constant composition and hence modulus of elasticity throughout.

An equation more accurately satisfying the experimental situation would include an allowance for the effect of change in shape of the beams as they bend. In practice, for engineering materials, this alters the deflection by something like 5% (Alexander, 1971). As Equation (1) compares two beams, which in this case are of similar dimensions and stiffness, these small effects on the radii of curvature of the glass fibre and the axopodium will tend to cancel each other out and so can probably be ignored. Finally, Equation (1) assumes that E_1 is the same during tension as it is for compression. There is no available evidence that bears directly on this point for microtubules. However, the two elasticities are the same for insect cuticle (Jensen and Weis-Fogh, 1962). In the experimental situation the strain in the axopodium and the glass fibre was small. This tends to minimise errors such as the last two described above.

CHAPTER V

FEEDING REACTIONS

Introduction

Heliozoans are voracious predators. They feed omnivorously on flagellates, ciliates, algae (Weston, 1856; Penard, 1904), and even small metazoans such as rotifers (Leidy, 1879), copepods (Dragesco, 1964) and gastrotrichs (Bovee and Cordell, 1971). Although Actinophrys accept a wide range of food organisms, Looper (1928) has shown that they exhibit a preference for small moving prey such as the smaller ciliates and flagellates. Size and strength of potential prey are two factors limiting the capture of food organisms by an individual. However, small groups of Actinophrys cooperate and subdue prey which one organism would be unable to capture alone (Looper, 1928). Because certain ciliates (e.g. Nassula sp.), which are evidently suitable as prey by criteria of size and strength, are not captured by Actinophrys (Looper, 1928) it is clear that other prey selection factors exist which have yet to be discovered.

It is generally agreed that food capture involves axopodia. Prey organisms which collide with axopodia adhere to them and so become relatively immobilised (Leidy, 1879). It seems possible that the adhesive is a product of the haptocysts. Opinion is divided as to whether or not a toxic paralysing effect is exerted on prey organisms (Penard, 1904; Kitching, 1960). Dragesco (1964) has summarised the evidence in this debate and concludes that material which causes organisms to stick to axopodia might also have a paralysing action which is more

effective against some species of prey organism than others. This opinion is supported by his observations that some organisms, which are passive during their capture, regain their activity when enclosed in a food vacuole.

Captured organisms move closer to the cell body of the heliozoan. There are several means by which this is accomplished. The most spectacular is by rapid contraction of the axopodia, which is described for the first time in this chapter. Small flagellates are "catapulted" towards the bases of the axopodia as a result of this feeding reaction.

When the prey is brought close (within about 30 μm) to the cell body, as a result of any of the possible mechanisms, it is engulfed. There is a rapid outgrowth of sheets of cytoplasm either from the base of an axopodium or from the cell body. These sheets of cytoplasm surround the prey and form a food vacuole. Digestion of the food proceeds within the vacuole and is normally complete within 8 hr (Penard, 1904). Undigested remnants can be expelled from the cell at any point on its surface in one sudden movement.

Results

(a) Feeding reaction to small flagellates

This feeding reaction is fairly specific. It occurs when three species of Ochromonas and one species of Chlamydomonas (see Materials and Methods) are captured and ingested. These flagellates are all roughly spherical in shape and have a diameter of about 10 μm. The reaction is not elicited by the pyriform ciliate Tetrahymena (length about 30 μm), nor does it occur in response to mechanical stimulations of various intensities with glass microneedles.

When one of the flagellates mentioned above contacts a single axopodium near its tip, the latter suddenly shortens in a fraction of a

second at a speed too rapid to be followed by the eye. The axopodium contracts from a normal length of 120-170 μm to a final length in the range 20-50 μm (Plate 45). The stump of the axopodium after contraction is no thicker along most of its length than it was previously, but it has a swollen tip, so that it resembles a drum-stick in shape (Plate 45). As the axopodium contracts, the flagellate is drawn down suddenly towards the cell body of the organism. Presumably the flagellate was attached to the axopodium at the point of initial contact. By the time the retraction is complete the flagellate is no longer attached to the axopodium. The flagellate remains stationary and is engulfed by a food-cup (Plate 46) which surrounds it more closely than the food-cups which surround ciliates prior to their ingestion.

Immediately after it has contracted, the axopodium starts to grow out again (Plates 51-53) at an initial rate of about 19 μm min^{-1} (Fig. 16). The swelling at the tip of the axopodium remains within a few μm of the tip but gradually becomes smaller and more pointed. Within 5 min of the start of growth, the swelling is no longer noticeable. As the axopodium grows longer its rate of elongation decreases. This aspect of the response will be dealt with in greater detail in the next chapter.

(b) Feeding reaction to the ciliate *Tetrahymena*

When the ciliate *Tetrahymena* encounters an axopodium it adheres to it. As the organism struggles thin strands of material can be seen connecting it to the axopodium and coating some of the ciliate's surface. Because similar material is seen on microneedles after micromanipulation experiments it seems likely that this material is a product of axopodia. *Tetrahymena* frequently become more enmeshed by their own struggles as they encounter more axopodia and so move nearer to the cell body. On other occasions, when they are passive, *Tetrahymena* move alongside axopodia towards the cell body slowly forcing neighbouring axopodia apart.

A cytoplasmic process grows out from a point near to the Tetrahymena on the surface of the cell body. It waves around in several directions apparently at random until it encounters the ciliate. The membrane then forms a food-cup which surrounds the ciliate (Plate 47) and fuses to form a food vacuole. Tetrahymena are often seen inside food vacuoles with cilia still beating at least 15 min after they have been engulfed. Occasionally Tetrahymena break free and escape. When this occurs food-cups do not immediately withdraw but continue their "blind search" for the departed Tetrahymena.

(c) Feeding reaction induced by inanimate material

To ensure that the means by which organisms such as Tetrahymena move closer to the cell body is not dependent on movement of the Tetrahymena themselves, Actinophrys was fed with inanimate material. Initially small latex particles (2.68 μm diameter) were added to culture medium which contained actively feeding organisms. These do not induce a feeding response in Actinophrys but are ingested by Tetrahymena as Mueller, Röhlich and Törö (1965) have noted. If Sephadex beads (diameter 10-40 μm) which have haemoglobin adsorbed to their surfaces (by immersion in a solution of about 5 crystals of haemoglobin dissolved in 10 ml culture medium) are introduced into ring preparations containing Actinophrys, they occasionally elicit a feeding response (Plates 48-50). The period from capture to ingestion lasts from 10-20 min, approximately twice as long as the period required to ingest a ciliate.

The Sephadex beads move slowly along axopodia in a centripetal direction. Occasionally axopodia appear to move apart as a bead approaches the cell body. A food vacuole is then formed in the normal way but it tightly encloses the Sephadex bead. Certain regions of the surface of the cell near to the point of ingestion are raised up (Plate 48) into small club-shaped processes which are about 5 μm in length. Within 1 hr

from the time of engulfment all the Sephadex beads have been egested.

(d) Egested material

Material egested from the cell body moves out along axopodia at a rate of about 60 μm min^{-1}. The distal movement ceases when such material reaches the tips of axopodia. On one occasion, a roughly spherical mass of this egested material was apparently attached to two axopodia because, as the material moved distally, the two axopodia were pulled together. After the material had moved about 20 μm both of the axopodia became freed and returned to their original straight form.

Discussion

(a) Rapid contraction of axopodia

The rapid contraction reported here takes place in a much shorter time (a fraction of a second) than any contraction previously reported for the axopodia of Actinophrys or its close relative Echinosphaerium. Other contraction rates reported for these heliozoans vary from a few seconds to several minutes (Bovee and Cordell, 1971; Tilney, 1971). Rapid contractions of a similar type have been reported for axopodia of the heliozoan Heterophrys, and the helioflagellate Ciliophrys (Davidson, 1969). The time taken for such contraction has been assessed for Heterophrys using high-speed cinematography. Axopodial contraction is completed in only 50 milliseconds (L. Davidson, personal communication). The arrangement of microtubules in the axonemes of Heterophrys (Bardele, 1970) is different from the "double spiral" pattern found in Actinophrys and Echinosphaerium.

Looper (1928) might have discovered this phenomenon had he had the advantage of present-day optics. He noticed that when Actinophrys feeds

on Euglena, the flagellates often make a "sudden jump" towards the cell body.

The shortening may involve virtually instantaneous breakdown of lengths of microtubules in excess of 100 μm for the following reasons. The shortened length of the axoneme is less than half the initial length and its base is covered by the nuclear envelope. The basal portion of an axopodium is no thicker after rapid contraction than it was before contraction took place. These factors make it unlikely that the mechanism is one of sliding or repacking of microtubules, for there is apparently not enough space for all the tubules to pack or slide into. Evidence concerning the rate of regrowth of the axopodium, which will be presented in Chapter VI, is also more consistent with microtubule breakdown rather than microtubule sliding.

The swelling at the tip of the contracted axopodium may possibly contain subunits released as a result of tubule breakdown. If so, there are clearly insufficient subunits contained in it to allow reformation of a whole axoneme because the swelling has disappeared long before the axopodium has grown out again completely. Furthermore, the volume of the swelling is less than the volume of the shortened portion of the axopodium. There are several reports that microtubules in basal portions of Echinosphaerium axopodia are more resistant to a variety of treatments than are the distal portions (for example, Shigenaka, Roth and Pihlaja, 1971). Therefore the conditions which suddenly induce microtubule breakdown are not necessarily restricted to the region distal to the stump of the contracted axopodium. A recent report has shown that lowering the concentration of Ca^{++} ions by the addition of E.G.T.A. favours the polymerisation of purified pig brain tubulin in vitro (Weisenberg, 1972). It is possible that some change, such as a sudden influx of Ca^{++} ions into the cytoplasm of the axopodium, promotes the extremely rapid breakdown of axonemes. Preliminary experimental applications of E.G.T.A. (see

Materials and Methods) to Actinophrys caused an unusual effect. Axopodia shorten by varying amounts but frequently also buckle at two or three points along their length. After this instantaneous reaction, axopodia remain in the same form without further loss of length for several minutes (Plates 90, 91).

It should be emphasised that work is done by the contracting axopodium because the flagellate is moved. The shortening is therefore a contraction, not simply a breakdown. If the external membrane of the axopodium is under tension it might act as an energy store for the contraction. Removal of axonemal microtubules would thus allow the membrane to contract drawing the distal portion of the axopodium nearer to the cell body. An alternative suggestion is that surface tension effects provide the energy for the contraction.

(b) Movement of objects alongside axopodia

It is possible that the rapid-contraction-feeding response is inadequate to capture prey which have a greater inertia than the small flagellates. This could be either because the force generated is not sufficient to move them or because the adhesive at the point of contact is not strong enough to withstand the strain of accelerating such a large object so rapidly. Since Ochromonas often become detached from axopodia during the axopodial contraction, the latter possibility seems the most likely.

One means by which Actinophrys transports larger prey to the cell body is occasionally seen during the Tetrahymena-feeding response. This mechanism is most convincingly demonstrated when inert particles are ingested. Centripetally directed "gliding movements" of prey alongside axopodia have been noticed previously but the transport mechanism involved is not understood (Kitching, 1960). Similar movements also occur in the opposite direction. Kitching (1962) noticed that certain treatments

induced a "skin" to lift off the cell surface and migrate out along axopodia at a rate of 20 μm min^{-1}. This "skin" was probably the product of the discharge of a large number of haptocysts. Egested material also moves distally along axopodia in a similar manner but at a faster rate (60 μm min^{-1}).

These movements are similar in some ways to the bidirectional streaming of the haptocysts along the axopodia within the cell membrane (rate of movement about 50 μm min^{-1}). It is possible that both types of movement are produced by the same mechanism. Consideration of the sequence of events which occur when a haptocyst discharges will make this clear. Each haptocyst is bounded by a membrane. The cause of haptocyst movement is not known; it might depend on an interaction between microtubules in the axoneme and the haptocyst membrane. In order for the haptocyst to discharge, it must first fuse with or penetrate the axopodial membrane and open to the exterior. Presumably, the contents of the haptocyst then spill out and cause the Tetrahymena to adhere to the haptocyst membrane. If this area of haptocyst membrane is still capable of movement directed by the same force that operated prior to discharge, the Tetrahymena will also move.

Other cases have been reported where membrane-bounded structures move parallel to the longitudinal axes of microtubules.

When algae are taken in by the ciliate Nassula (Tucker, 1968), they are enclosed within membrane-bounded invaginations of the cell membrane. Algae move into these organisms through complex microtubular feeding organelles called cytopharyngeal baskets. Kubai and Ris (1969) have shown that in the dinoflagellate Gyrodinium chromosomes move to the poles of nuclei within an intact nuclear envelope during nuclear division. The chromosomes form V-shaped configurations in the nucleoplasm adjacent to the nuclear envelope walls which surround tunnels of cytoplasm which extend from one pole of the nucleus to the other. These cytoplasmic

tunnels contain spindle microtubules.

If there is a single force-production system of this type which causes movements alongside axopodia it could also be responsible for the separation of free-floating daughter Actinophrys during binary fission (Chapter II).

(c) Stimulation of food-cup production

Kitching (1960) investigated the formation of food-cups in Actinophrys. He concluded that the most effective stimulus for food-cup production is a combination of chemical and mechanical stimuli. The observation that, once formation has been stimulated, a food-cup will go on extending for several minutes after the prey has escaped, indicates that this reaction once initiated is not closely controlled.

CHAPTER VI

COOLING EFFECTS AND RECOVERY FROM COLD TREATMENT

Introduction

A reduction in temperature (below about 4°C) causes the breakdown of microtubules of many types. It is more damaging to certain types of cytoplasmic tubules than to those of cilia and flagella (Behnke and Forer, 1967). Heliozoan axonemal tubules behave like the former type; they break down and the axopodia of Echinosphaerium shorten when these organisms are cooled to 4°C. (Tilney and Porter, 1967).

Prior to the study presented here, no information about the rate of outgrowth of axopodia in organisms recovering from low temperature treatments was available. The two pioneering electron microscopic studies of the process of microtubule pattern reformation which have been made, in which organisms were fixed at timed intervals during the reformation of axonemes of the heliozoans Echinosphaerium and Raphidiophrys from low temperature treatment, allow few conclusions to be drawn (Tilney, 1967; Tilney and Porter, 1971; see also Discussion). Thus in an attempt to come to a fuller understanding of the processes of axopodial outgrowth, axonemal length determination, microtubule initiation, and microtubule pattern formation, a study of the recovery of Actinophrys from various cooling treatments was undertaken.

Results

(a) Outgrowth of axopodia after cold treatment

Changes in the lengths of axopodia are believed to represent

changes in the lengths of their microtubular axonemes because axonemes invariably extend the full length of axopodia.

Organisms maintained at $0^{\circ}C$ for 45 min possess fewer axopodia than organisms at room temperature ($20^{\circ}C$). These axopodia shorten from initial lengths which are often greater than $150\mu m$ to lengths which are usually less than $30\mu m$ (Plate 54). Extending the period of treatment at $0^{\circ}C$ to 4 hr produces no further substantial shortening of axopodia.

To analyse the recovery of organisms from cold treatment, preparations were removed from containers of melting ice and placed on the stage of the microscope. Thermocouple readings indicate that the small drops of culture medium containing organisms warm up, attaining a stable final temperature of $21^{\circ}C$, which is about $1^{\circ}C$ higher than room temperature ($20^{\circ}C$), within about 4 min (Fig. 17). The axopodia of organisms in such preparations start to increase in length immediately after removal from cold (Fig. 17). The rate of outgrowth reaches a maximum (approx. $10\mu m\ min^{-1}$) about 4 min after removal from the ice container. For some time after this, outgrowth continues at a uniform rate of about $6\mu m\ min^{-1}$ (Fig. 17).

Once its axopodia have grown to a certain length, an organism's normal rolling movement (Watters, 1968) recommences. This makes it impossible to obtain accurate measurements of the variation in length of single axopodia over long periods. Assessments of outgrowth rates over relatively long periods (greater than 20 min) can be made by plotting the average lengths of longest visible axopodia in several organisms at various times after termination of the cooling treatment (Fig. 16). Apparent instances of shortening in Figure 16 may be explained by the variations in length between axopodia which are often quite marked during the outgrowth of an organism's axopodia after cold treatment. Using average length measurements, the rate of outgrowth of axopodia appears to decrease as axopodia become longer (Fig. 16).

FIGURE 16

Shows changes in the lengths of axopodia elongating (at 20°C) after cold treatment. Each point on the graph represents the mean length of the longest axopodia observed in four organisms.

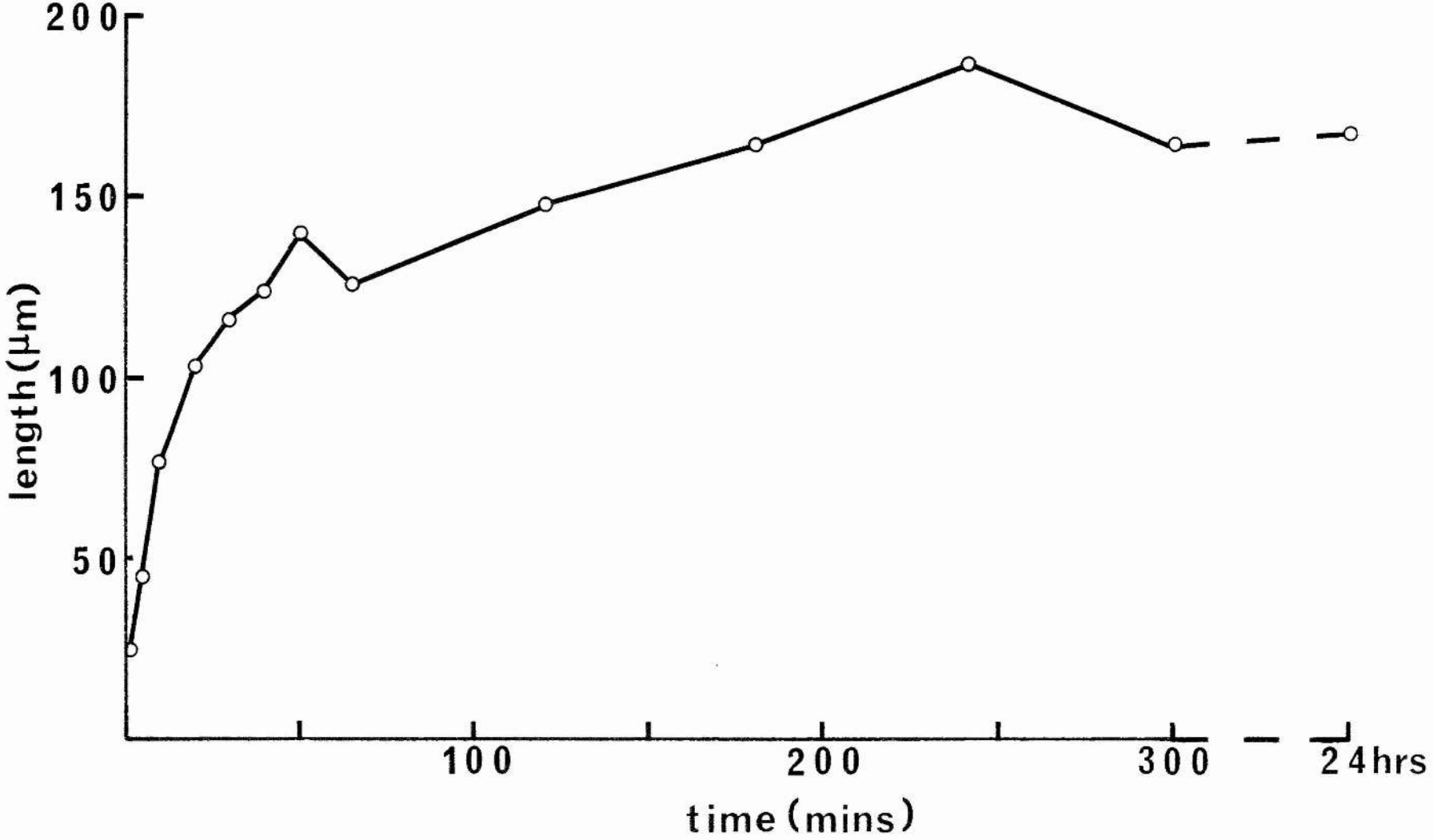
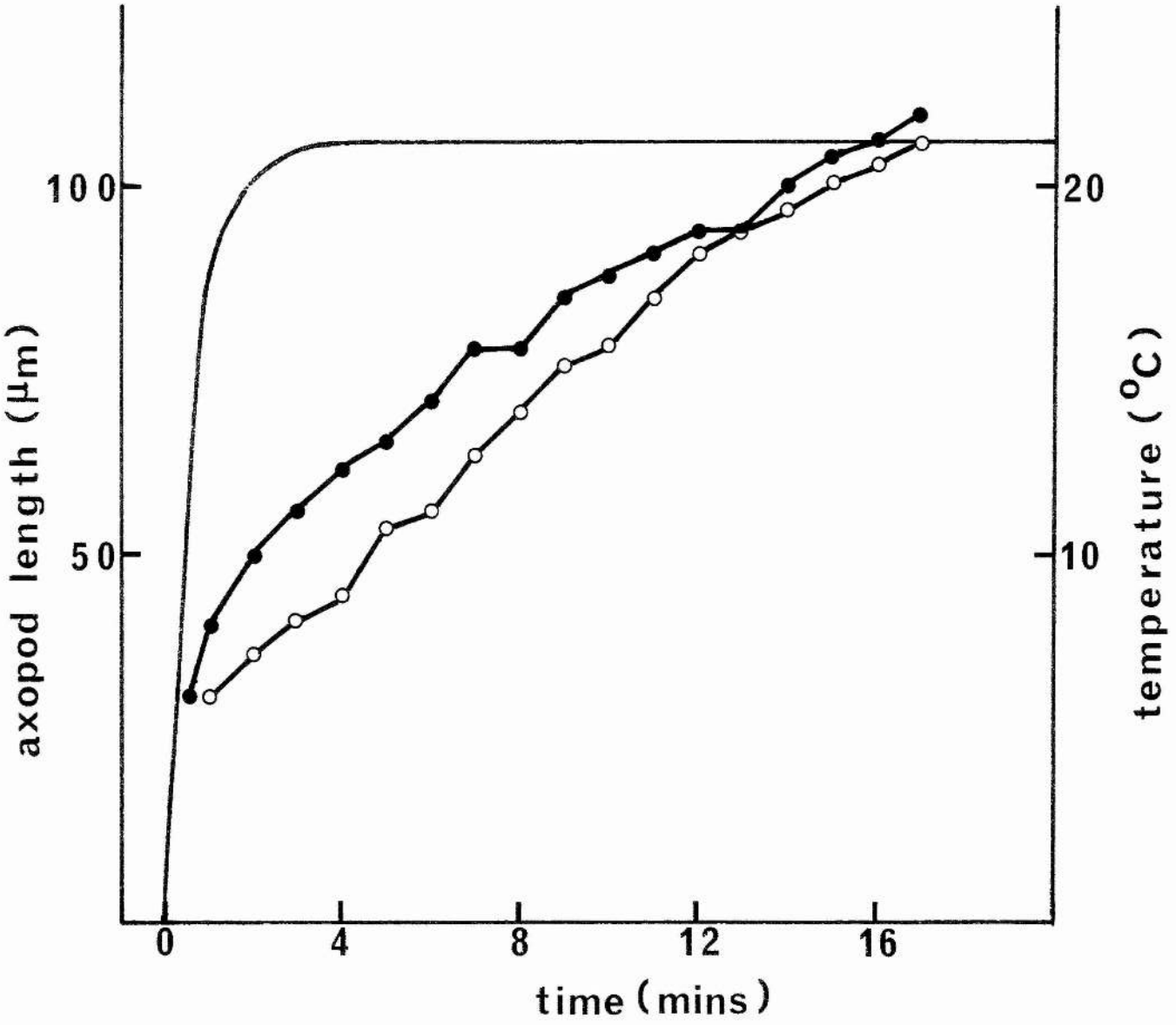

200
150
100
50
length (μm)
100
200
300
24hrs
time (mins)

FIGURE 17

Shows changes in the base to tip lengths of an axopodium elongating after rapid contraction (at 20°C) (black circles) and another axopodium elongating after cold treatment (open circles). The increase in temperature of the culture medium after cold treatment is also shown (line without circles).


100
50
axopod length (μm)
20
10
temperature (°C)
0
4
8
12
16
time (mins)

The rate of outgrowth of an axopodium which has retracted during feeding on a small flagellate is shown (Fig. 17) to be similar to the rate of outgrowth of axopodia after retraction in response to cold. The difference in the initial rates of outgrowth is probably due to the lower temperature (during the first 4 min) in the preparations which contain organisms recovering from cold. A comparison of recoveries can therefore be made between a situation where a single axopodium has been retracted (after feeding) and a situation in which all the axopodia (more than 100) have been shortened (after cold).

(b) Mild cooling of axopodia

Even if organisms are only cooled slightly, to about 15°C, their axopodia shorten. The longest visible axopodia of organisms increase in length on average by about 40% on rewarming to room temperature (20°C) after only 1 hr at 15°C in a cooled incubator. However, the average length (126 μm) of 100 axopodia measured from a total of 20 Actinophrys maintained at 15°C for 48 hr is almost the same as the average length (128 μm) of a similar sample of axopodia measured for organisms maintained at room temperature (20°C) throughout the 48 hr period.

(c) Regrowth of axonemes after cooling

Unlike the situation in Echinosphaerium reported by Tilney and Porter (1967), the axopodia of Actinophrys do not all retract completely even after 12 hr at 4°C. The best conditions for achieving extensive retraction of axopodia in Actinophrys without causing permanent damage to the organisms has been determined (Materials and Methods). After 6 hr at -3°C most of the axopodia have retracted completely, and grow out again when organisms are returned to room temperature. After longer periods at -3°C axopodia do not recover and organisms eventually die.

Such organisms were fixed for electron microscopy at intervals after

their removal from the cooled incubator to room temperature (18°C; Table VII). The solution of glutaraldehyde used for the initial stages of fixation was chilled to the same temperature as the Actinophrys, to ensure that the very earliest stages in the axonemal reformation were obtained. The temperature of the pre-fixative and the culture medium containing the Actinophrys was measured with a thermocouple for each timed fixation (Table VII).

A control fixation of Actinophrys which have already been recovering for 2 min is made using fixative at -3°C. This is necessary because the absence of microtubules or patterned aggregates of microtubules at any time within the first 2 min of recovery may result either from the lowered temperature of organisms or as an artefact caused by the use of cold fixative.

TABLE VII: The temperature of organisms and fixative at intervals after removal from cold

Time after termination of cold treatment (min)	Temperature of Actinophrys (°C)	Temperature of glutaraldehyde (°C)
0	-3.0	-3.0
1	4.2	-1.0
2	7.6	0.8 (-3°C)
5	13.1	4.2
10	15.8	7.3
45	18.0	14.9

Because there was a risk of Actinophrys dying and hence through degeneration exhibiting altered ultrastructure a sample of organisms was taken from the culture of Actinophrys immediately after the cooling treatment. These organisms were allowed to recover at room temperature and after 50 min they were examined. There was no mortality and without exception the organisms possessed axopodia which appeared to be growing out in the usual way.

Analyses of the changes in Actinophrys during recovery from this

cooling treatment were made using thin sections taken from organisms fixed at intervals after removal from the cold to room temperature. Three organisms were sectioned for each stage in the recovery from cold (Plates 55-60 show the stages in the recovery of a living organism from 6 hr cold treatment at -3°C).

(i) Axonemal microtubules in organisms after 6 hr at -3°C

No axopodia protrude from the cell body. A very few axonemal remnants are still present in which the usual pattern of microtubules is still just recognisable (Plate 62). Axonemes which remain are broken down most completely at their tips. The number of axonemal vesicles in these Actinophrys is much greater than it is in organisms fixed at room temperature. They often appear to be clustered close to axonemal remnants (Plates 63, 64).

Far fewer microtubules are present than in organisms fixed at room temperature. Microtubules which remain are not usually straight. Most have lost their radial orientation, and for all of them the density of their walls is much less than is usually the case. Few microtubules are attached to the nuclear envelope (Plate 61). The nucleus of these cells is almost always eccentrically placed.

(ii) One minute after cold treatment

The most noticeable difference in structure between this stage and the last is the vast increase in the number of microtubules (Plate 66). These are most commonly found near or attached to the nuclear envelope. They do not appear to have a precise radial orientation (Plate 65). The points at which tubules contact the nuclear envelope are not grouped together as would be expected if the tubules of an axoneme had simply "fallen apart" (compare Plate 67 and Plate 81). One or two well-ordered medium-sized axonemes are seen in organisms at this stage (Plate 67). These may represent stages in the recovery of the axoneme remnants still

present in organisms after 6 hr cold treatment. Medium-sized axonemes apparently never contain more than four turns of the "polygonal spiral pattern" (i.e. they contain less than 76 tubules).

(iii) Two minutes after cold treatment

Most microtubules occur individually (Plate 68). Groups when they occur are not radially oriented. Profiles of microtubules in longitudinal section appear longer than profiles of tubules in organisms after 1 min recovery from cold (Plate 69). Medium-sized axonemes are more common than in organisms after 1 min of recovery but each axoneme appears to contain approximately the same number of tubules as in organisms after 1 min of recovery from cold. Medium-sized axonemes frequently occur in the outer region of the endoplasm at times when no such patterned aggregates of tubules are present close to the nuclear envelope. Groups of axonemal vesicles frequently occur adjacent to axonemes (Plate 68).

(iv) Organisms fixed after two minutes recovery from cold treatment with fixative at -2°C

The ultrastructure of these Actinophrys is indistinguishable from that of the organisms fixed at the same stage of recovery using warmer fixative (Plates 71-73). Hence the difference noticed between structures at different recovery stages represents a real change occurring in the recovery process, not an alteration caused by the application of fixatives at different temperatures.

(v) Five minutes after cold treatment

No axopodia are present (Plate 76) but several well-ordered axonemes are present in the cell body (Plate 77). These are frequently still not oriented in a radial manner (Plate 77). In general axonemal pattern is less well defined in the region close to the nuclear envelope than it is in the remainder of the endoplasm; tubule attachment points on the nuclear envelope are apparently randomly arranged (Plate 76). Small clusters of

microtubules which lie parallel to each other, and which are not packed in the usual axonemal pattern, are common throughout the cytoplasm (Plates 74, 75). Occasionally C-tubules (Cohen and Gottlieb, 1971) are seen in these groupings (Plate 75).

(vi) Ten minutes after cold treatment

At this stage the first signs of axopodial outgrowth are detectable. Small axopodial protrusions occur which contain a few widely-spaced microtubules interspersed with some dense amorphous material (Plate 82). Many axonemes are now radially oriented, and some have terminal contacts with the nuclear envelope (Plates 80, 81). More medium-sized axonemes occur than in organisms which have been recovering for 5 min. Certain of these axonemes exhibit patterns of tubule packing which differ from the usual pattern of microtubules in axonemes (Plates 78, 79). Fewer microtubules are present than is the case in material fixed at earlier stages of recovery.

(vii) Forty-five minutes after cold treatment

After 45 min of recovery from cold, organisms possess more axopodia than after 10 min of recovery. Most of them are longer than the axopodia of organisms which have only had 10 min for recovery. The axopodia contain haptocysts, mitochondria, and more microtubules than at earlier stages of recovery (Plate 84). Axonemal tubules apparently extend the full lengths of axopodia (Plate 84). Transverse sections of axopodia show that the microtubules of the axoneme are not packed in the usual way. They exhibit both gaps and deviations from the usual axonemal pattern (Plate 83). No axonemal vesicles are present in the distal portions of growing axopodia; they still occur commonly in regions surrounding axonemes within the cell body (Plate 85).

At this stage the nuclei of most organisms are centrally positioned. Certain axonemes can be traced continuously from the surface of the

nucleus to the external cell membrane. Nearly all of the axonemes are radially oriented. Some axonemes are larger, 5 turns of each polygonal spiral (i.e. 118 tubules) being present, than the largest axonemes found in organisms fixed after shorter recovery periods. However, recovery is still not totally completed (Plates 86, 87), because many tubules still occur which are not radially oriented and are not packed in the axonemal pattern.

(viii) Summary of sequence of events in recovery from cold treatment

(1) Short microtubules appear near the nucleus.

(2) Microtubules grow longer.

(3) Microtubules aggregate into bundles.

(4) Bundles of tubules gradually achieve the correct axonemal packing.

(5) Bundles become radially oriented.

(6) Axonemal bundles are attached at their bases to the nuclear envelope.

(7) Nuclei have moved to a central position in the cell body.

(8) Axopodia begin to grow out.

The results presented above reveal that many of these processes are continuous and occur simultaneously. This sequence is merely intended to indicate an order in which events appear to be initiated during the recovery period.

Discussion

(a) Rate of axopodial outgrowth

The only other study on the rate of axopodial outgrowth in heliozoa (Shigenaka, Roth and Pihlaja, 1971) shows that in Echinosphaerium,

after dilute urea treatments, the rate of extension of axopodia decreases as the axopodia become longer. A similar pattern of outgrowth has been noticed in flagellar regeneration studies, undertaken by Rosenbaum and Child (1967) and Tamm (1967). Since the present study and the three cited are in such good agreement, all narrow cell outgrowths, which contain microtubular axonemes, may grow out in this way. The underlying reason for this gradual decrease in the rate of extension is probably of cardinal importance to an understanding of how the lengths of microtubular structures are determined.

Electron microscopic study of retraction of axopodia in Actinophrys after cold treatment shows that this process involves the breakdown of axonemal microtubules. Because the rate of outgrowth of a single axopodium after the flagellate feeding contraction is approximately the same as the rate of outgrowth of axopodia after cold treatment it seems likely that cold-treated axonemes have been broken down to a similar extent during retraction (see Chapter V).

Organisms after cold treatment (where all the axonemes retract) contain more microtubule subunits than organisms which just retract one axoneme during the flagellate-induced feeding contraction. If these subunits enter a common cytoplasmic pool, and since the rates of outgrowth of axonemes in about the first 15 min of recovery are similar (Fig. 17), it appears that the concentration of microtubule subunits might not be rate-limiting during this early stage of outgrowth.

There is some equivocal evidence that flagellar microtubules grow by the addition of material at their tips (Rosenbaum and Child, 1967). If this is also the case for the microtubules of heliozoan axopodia then the rate of outgrowth at later stages could be limited by several factors. In this case the concentration of tubule subunits could well become limiting because of some factor such as the difficulty of transporting subunits to the remote tips of the axopodia. Alternatively, localised

differences in conditions at the tips of axopodia such as variations in the concentration of certain ions in the cytoplasm could be responsible for the reduced rates of outgrowth. It has been reported that the *in vitro* reconstitution of microtubules from subunits is favoured by the presence of Mg^{++} ions and the removal of Ca^{++} ions (Weisenberg, 1972).

The early rate of outgrowth of microtubules in axopodia after cold treatment (about $10 \mu m\ min^{-1}$) is faster than the rate of natural axopodial growth ($4 \mu m\ min^{-1}$ — Chapter II), and the rate of increase in length (4-5 $\mu m\ min^{-1}$) of the birefringent astral fibres of *Psammechinus* (Swann, 1951). At least 50% of spindle fibre birefringence is due to microtubules (Forer, 1969). This implies that under conditions of normal growth (not regeneration after retraction) some other factor or factors are rate limiting. One of these extra factors could be the concentration of microtubule subunits.

(b) The "nucleation" of microtubules

The recovery of *Echinosphaerium* (Tilney and Porter, 1967) and *Raphidiophrys* (Tilney, 1971) from cold treatments has been studied electron microscopically. Because rates of early axopodial outgrowth reported here are so fast it appears that Tilney used inappropriately long time intervals between his fixations. In both his studies the first "recovery fixation" was made 10 min after the start of the recovery period. Using *Actinophrys* axopodial outgrowth rates as an approximate measure of the rate of microtubule growth in heliozoans it appears that the tubules Tilney described in the first stage of recovery might already have been $100 \mu m$ in length. It is apparent that Tilney's evidence cannot adequately resolve the precise site of microtubule nucleation. Even the evidence presented here showing some microtubules attached to the nuclear envelope of *Actinophrys* within 1 min of the onset of recovery is equivocal. The statement that most microtubules are nucleated close to the nuclear

envelope is the only conclusion present evidence allows. Indeed the presence of badly-oriented yet well-developed axonemes and axopodia (Plate 77) suggests that formation of contact with the nuclear envelope in certain axonemes may be delayed until several minutes after termination of cold treatment.

(c) Models for axonemal pattern determination

In Actinophrys microtubules appear to be nucleated as a distinct process, which is separate from that of axonemal pattern determination. It is clear, therefore, that any model which combines these two features (e.g. Tucker, 1970) is inadequate as an explanation of these events. In other situations, for example morphogenesis of the cytopharyngeal rods in Nassula (Tucker, 1971), and the development of flagella in Allomyces (Renaud and Swift, 1964), it may be that the model is adequate.

At present, there is not sufficient evidence to make a judgement on two other models which suggest that axonemal pattern formation is a self-assembly process and not a product of a "pre-pattern" on a template of some kind. The essential distinction between these other models is that Tilney and Byers (1969) suggest that pattern is determined because cross-links attach to particular subfibres in the microtubule wall, whereas Roth et al. (1970) suggest that pattern is determined because any subfibre has the potential to bind the first cross-link, but thereafter the likelihood of further cross-links being formed is increased at certain sites because of long-range allosteric changes.

Biochemical isolation of heliozoan axonemal tubulins could throw light on this problem. If different tubulins were to be found in different subfibres around the tubule wall, as has been shown recently to be the case for subfibres in the outer doublet tubules in Chlamydomonas flagella (Witman, Carlson and Rosenbaum, 1972), it would favour the argument put forward by Tilney and Byers.

(d) Model for the orientation and siting of axonemes

Axonemes bind terminally (Plate 2) and laterally (Roth et al., 1970) to the nuclear envelopes of Actinophrys and Echinosphærium respectively. They also occur frequently at one cross-link's length from haptocyst membranes (Roth et al., 1970) and are often aligned adjacent to the membranes of large ectoplasmic vacuoles (Plate 77). It is possible that binding to membranes increases the stability of axonemes. Axonemes in certain situations which have maximal membrane contact (for example at the meeting point of several ectoplasmic vacuoles) might be more stable than axonemes in other situations. Thus it might be that the orientation and number of axonemes in an organism is partly determined by the arrangement and nature of the large vacuoles that constitute most of the ectoplasm. The substitution of D_2O for water causes microtubules to become more stable (Marsland and Zimmerman, 1965; Inoué, 1964). However, D_2O also causes a reduction in the length of the axopodia of Echinosphærium (Marsland, Tilney and Hirshfield, 1971). Although the authors do not mention it, this treatment also results in a disturbance of the orderly array of ectoplasmic vacuoles (Marsland et al., 1971; Fig. 1). It might be that more "favourable sites" for axoneme formation are created as a result of this disturbance of the ectoplasm. More microtubules, in more axonemes, might still occupy shorter axopodia than is normally the case. Thus the apparently paradoxical observation of Marsland et al. (1971) could be resolved. It is pertinent that Echinosphærium from old-established cultures contain more, shorter axonemes than organisms from freshly inoculated cultures (Roth et al., 1970).

(e) The effect of cooling on microtubules

The polymerisation of Tobacco Mosaic Virus (T.M.V.) protein is favoured by increased temperature and the application of D_2O, but is inhibited by reduced temperature. The interpretation of this and other

evidence has led to the suggestion that hydrophobic bonding is involved in the polymerisation process and that during polymerisation bound water is released (Stevens and Lauffer, 1965; Lauffer, 1966; Khalil and Lauffer, 1967).

Because of the obvious structural analogy with T.M.V. and because the polymerisation of tubulins is affected in the same way by the treatments mentioned above, Marsland et al. (1971) suggested that tubule polymerisation might take place in a similar manner to T.M.V. polymerisation. There is apparently no evidence against this possibility.

As would be expected most chemical bonds are stabilised as lowered temperature reduces the molecular movements which tend to break bonds. Kauzmann (1959) however has shown that hydrophobic bonds are destabilised by a reduction in temperature. This is probably because a reduction in the motion of water molecules which surround the protein reduces the repulsive force exerted on unbound hydrophobic surfaces on the protein molecules. If, when microtubules are cold treated, they break down to the dimer form, it is probable that hydrophobic bonds are involved in the polymerisation process. However, an alternative possibility is suggested by the increase in axonemal vesicles as axonemal microtubules break down. If the free "dimer" is the highest energy state of tubulin slight cooling (down to about 20°C) could lead to the stabilisation of dimers into the cross-bonded microtubular form. Further cooling (down to about 0°C) may stabilise parts of the tubule wall into yet more stable aggregates (the axonemal vesicles). The coiled appearance of these bodies (Plate 69) suggests some potential for cross-bonding. If this is the real situation there need not be a major involvement of hydrophobic bonding.

(f) Determination of the length of microtubules

The simple assumption, that microtubule polymerisation terminates, and tubule length becomes fixed, when a pool of microtubule subunits is

exhausted, can explain certain phenomena. Heliozoans from old-established cultures possess more axonemes than organisms from younger cultures, yet these axonemes are shorter than is normally the case (Roth et al., 1970). In the ciliate Nassula when the number of certain microtubule bundles is experimentally reduced they grow to greater than normal lengths (Tucker, 1971a).

The observation that axopodial outgrowth rate decreases as axopodia lengthen might indicate that the rate of microtubule lengthening is tubulin concentration dependent. It might also suggest that subunits bind less readily to long tubules than to short ones. Such a situation could arise through some graded sequence of confomational changes in subunits along the length of tubules such as that suggested by Roth et al. (1970).

Microtubules may be in a dynamic state in which subunits are being continually added and lost at their tips. Tubule elongation could then cease when the rates of loss and gain of subunits were equal. Tilney (1968; 1971) has presented evidence for a dynamic interaction of this kind between the pool of subunits and the microtubules; he has also considered the extent to which the equilibrium position is sensitive to treatments with cold, colchicine and deuterium oxide. However, the axonemes on an organism vary in length. Further axonemes in axopodia which have contracted as a result of the feeding response to flagellates increase in length by 100 μm while no similar increase in length occurs in adjacent axopodia. Such considerations suggest that other factors, which have not previously been considered in heliozoa, influence axonemal length determination. Some form of localisation of subunits, or of other materials which inhibit or promote axonemal assembly, might be responsible.

It is also clear that some long-term control over the length of axonemes is exercised, because after 48 hr at 15°C axopodia are the same length as axopodia at 20°C whereas after only 1 hr at 15°C they have

decreased in length by 40%. This may be accounted for by synthesis of axonemal constituents which results in an increase in the size of the pool(s).

CHAPTER VII

IRRADIATION OF AXOPODIA WITH ULTRA-VIOLET LIGHT

Introduction

Ultra-violet irradiation has been used extensively to disrupt the microtubule containing spindle fibres of dividing cells (Zirkle et al., 1960; Inoué, 1964). The mechanism of action of ultra-violet light on these components has been discussed by Forer (1965). He discounted the possibility that the proteins of the fibre were denatured and showed that the action was one of partial or total disaggregation of oriented protein molecules.

What determines the length of axonemal microtubules? As an approach to this problem ultra-violet irradiation is a technique of great potential value. Such irradiation might interfere with the postulated equilibrium between axonemal microtubules and their precursor subunits. Furthermore, since the size of ultra-violet light microbeams can be reduced to cross-sectional areas of $4\,\mu m^2$ (Smith, 1964), a microbeam can be used to irradiate a single axopodium. Consequently a study of axopodia was undertaken using this equipment.

All the following experiments were performed at a room temperature of about $25^{\circ}C$ with an ultra-violet microbeam which had a radiation intensity of 10^{-3} ergs/μm^2/sec.

Results

(a) Survival of organisms

Actinophrys which have not been irradiated usually survive for at

least 5 hr in the type of preparation used (Fig. 4). If a square beam of ultra-violet light of side 26 μm is directed at the cell body of an organism for 1 min (a dose of 6 x 10^{-2} ergs/μm^2), the organism usually breaks open. When this happens the axopodia in the beam are destroyed yet unirradiated axopodia are apparently undamaged. In fact even after the extrusion of nuclei and some cytoplasm long straight axopodia are visible for at least 30 sec.

(b) Irradiation of isolated nuclei

The resistance of the nuclear envelope to ultra-violet irradiation is of interest in view of the fact that nuclear membranes may be composed partly of tubulins (Stadler and Franke, 1972).

The isolated nuclei of burst cells are resistant to continuous irradiation for 10 min at the same radiation intensity that causes the cell membrane to burst after 1 min. Although this result apparently yields no information about materials composing the nuclear envelope, it is consistent with unpublished observations that a variety of physical and chemical treatments break down the cell membrane whilst the nuclear envelope, which has the unusually complex structure described in Chapter I, remains intact.

(c) Irradiation of different parts of the organism

Irradiating tips of axopodia at a rate of 8.3 x 10^{-2} ergs/sec for 10 sec causes the disintegration of the tips of the axopodia. Irradiation for 8 sec causes a different effect: no visible parts of axopodia break off yet the axopodia shorten. This effect will be termed retraction. This retraction continues during the minute after irradiation has ceased. It is accompanied by the centripetal passage of small "bulges" along axopodia towards their bases.

When an axopodium is irradiated approximately half way along its

length at a radiation intensity of 8.3×10^{-2} ergs/sec for 10 sec the whole portion of the axopodium distal to the irradiation area breaks off. This effect will be termed amputation. At similar points along the length of other axopodia, 8 sec of irradiation at the same intensity has no apparent effect.

Axopodia can be amputated at their bases with exposures to ultraviolet irradiation of from 10-20 sec at a radiation intensity of 8.3×10^{-2} ergs/sec. If the beam is "aimed" in such a way that it partly covers the base of an axopodium yet overlaps onto the cell body, the axopodium is usually amputated after irradiation for about 15 sec. If the irradiation is continued, after the axopodium has been lost, for a further 75 sec, that part of the cell surface directly under the beam is pulled back into the cell body. If the microscope stage is moved immediately so that this area does not "escape the beam", rupture of the cell membrane occurs after a further 30 sec of irradiation.

(d) Axopodial regrowth after irradiation

Tip irradiation, of the type described which caused retraction without loss of material from axopodia, was undertaken. The lengths of single axopodia treated in this way were measured at 1 min intervals from the time of irradiation until the rolling movement of the organisms interfered with the measurements. During the first 2 min after treatment the axopodia shorten at an approximately uniform rate of about 17 μm min^{-1}. Axopodia then grow out immediately at an average rate of about 5 μm min^{-1}. A typical record of such retraction and outgrowth of an axopodium is shown in Figure 18. In six experiments the outgrowth rate varies between 3.5 and 8 μm min^{-1}. Outgrowth rates were also assessed for axopodia which had been shortened to half of their original lengths because the distal portion was amputated. Measurements made at 1 min intervals after amputation show that there is no comparable decrease in

length after irradiations have ceased, but outgrowth commences immediately at a uniform average rate of $2\,\mu m\ min^{-1}$ (Fig. 18). The range of outgrowth rates in seven experiments of this type varies between 1 and $4\,\mu m\ min^{-1}$.

The rate of outgrowth of an axopodium during the first ten minutes of recovery, when it has been amputated once, is faster than the rate of recovery in the ten minutes following immediate reamputation for a second time. Similarly this latter rate of growth is faster than the rate of growth following immediate reamputation for a third time.

(e) The effect of axopodial length change on neighbouring axopodia

In these experiments an axopodium was selected and measured. As many other axopodia in its vicinity as possible were then amputated using the microbeam. At intervals thereafter, the lengths of both the unirradiated axopodium and the "stump" of an adjacent amputated axopodium were recorded. The result (Fig. 19) of an experiment of this type shows that the amputated axopodium grows out at a rate of $2.5\,\mu m\ min^{-1}$. Simultaneously the unirradiated axopodium shortens, initially by up to $20\,\mu m$, and then grows out to regain its initial length.

Unfortunately it was impossible for technical reasons to perform a similar experiment to this where other axopodia were induced to retract whilst a non-irradiated axopodium was monitored for possible length increases.

Discussion

(a) The effect of ultra-violet irradiation

The large difference in effects between amputation and induced retraction, which depend on only a 2 sec difference in irradiation time and a small alteration in target site, could be due to several factors.

FIGURE 18

Point A indicates the initial length of an axopodium. Line A shows the changes in length of this axopodium as it shortens and grows out after mild irradiation with an ultra-violet microbeam at its tip. Point B shows the initial length of another axopodium. Line B shows the changes in length of the basal portion of this axopodium as it grows out after amputation at a point approximately half way along its length by an ultra-violet microbeam.

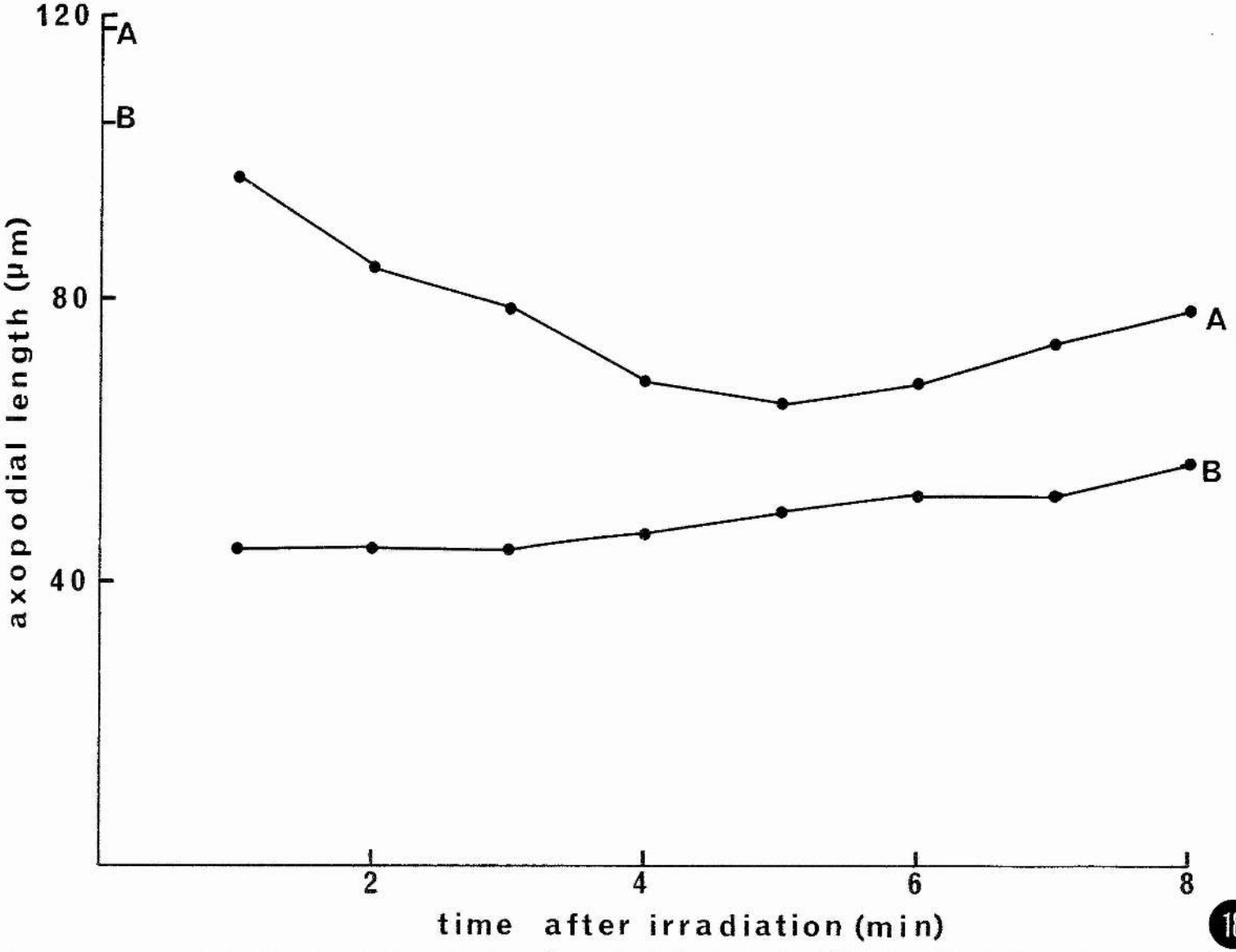
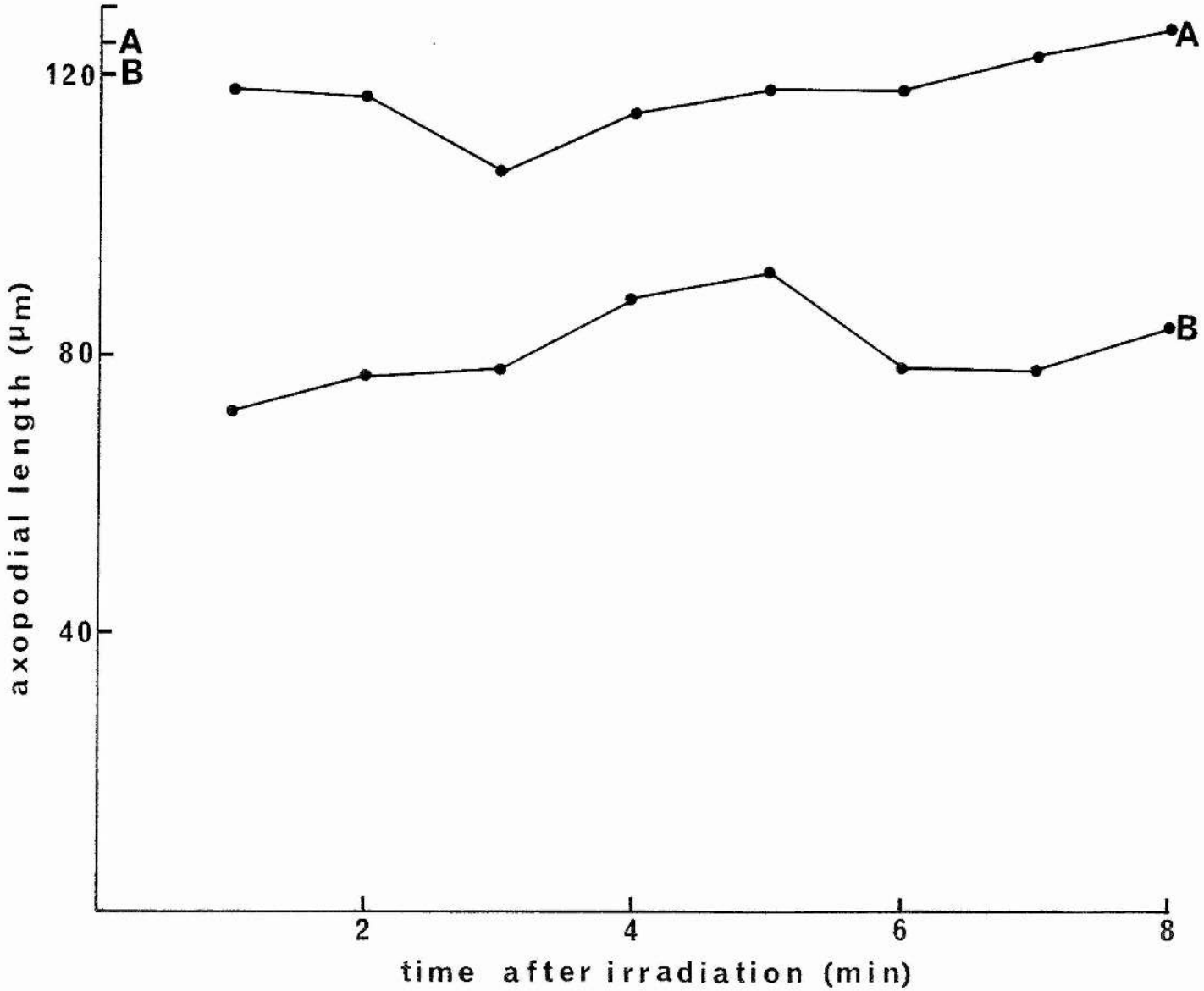

120
A
B
80
40
axopodial length (µm)
A
B
2
4
6
8
time after irradiation (min)

FIGURE 19

Point B is the initial length of an axopodium. Line B shows the changes in length of this axopodium after amputation (approximately half way along its length) with an ultra-violet microbeam. Several axopodia on this organism were amputated in this way. One axopodium (initial length shown by point A) was not irradiated with the microbeam. Changes in its length are shown in line A. The axopodia whose length changes are recorded in lines A and B were adjacent to each other.


A
B
120
80
40
axopodial length (µm)
A
B
2
4
6
8
time after irradiation (min)

But, because proteins absorb ultra-violet irradiation (maximally at 225 mμ but to a lesser degree at other wavelengths), it seems likely that one fundamental effect is the breakage of chemical bonds between structural molecules as a result of an increase in the energy of axopodial proteins.

When mild terminal irradiation of axopodia ceases, axopodia continue to contract. Swellings apparently move down the axopodia during this contraction; it is possible that a similar situation to that which results from colchicine treatment is operative (Chapter III). Microtubules, in this situation also, appear to be breaking down from their tips.

Since no electron microscopy has been performed on spindle fibres after they have been exposed to ultra-violet irradiation (Forer, 1965), these observations of amputations and induced retraction of microtubular axopodia are of general interest. They support the interpretation that the action of ultra-violet light on spindle fibres is at least partially owing to a destructive effect on microtubules.

(b) The rate of axopodial outgrowth

When axopodia retract without loss of material the regrowth rate is about 5 $\mu m\ min^{-1}$ at 25°C. Over a similar range of axopodial lengths, outgrowth after cold treatment and rapid contraction takes place at rates of approximately 5 $\mu m\ min^{-1}$ and 4 $\mu m\ min^{-1}$ respectively. Although these latter measurements were made at 22°C (3° lower than the temperature at which the ultra-violet irradiations were performed), the rates are sufficiently similar for there to be little question of permanent injury to the axopodium as a result of irradiations at this dose level.

The possibility remains that the slower rate of outgrowth of amputated axopodia is an indirect effect caused by damage to some system indirectly related to the growth of the axoneme. However, this slower rate is also consistent with the theory that the rate of growth of axopodia over this length range is limited by the supply of some material, possibly

to the postulated tip growth zone of the axoneme. This latter type of interpretation is supported to some extent by the observation that there is a reduction in outgrowth rate of axopodia which suffer sequential amputations. In successive amputations, the damage is probably the same on each occasion, and yet the total amount of microtubule protein which the organism has lost increases each time. This correlates well with the reduction in the rate of outgrowth.

This situation is not comparable to that described by Rosenbaum and Child (1967). They found that the regrowth of protozoan flagellae after a first amputation followed the same pattern of rates as they did during "re-regeneration" after a second amputation. In that situation a lag period during which synthesis of new components could take place was available to the organism.

(c) The effect of axopodial length change on neighbouring axopodia

This experiment owed a great deal in its design to an experiment performed by Coyne and Rosenbaum (1970). They studied the effect of amputating the distal half of one flagellum from the biflagellated Chlamydomonas. During the initial part of the recovery period the longer of the two flagella shortened whilst the shorter one lengthened. After the flagella had reached approximately equal lengths they both started to grow outwards together at the same rate. The authors' interpretation of this experiment is that axonemal protein from the shortening flagellum passes into a common pool and thus becomes available for lengthening of the amputated flagellum.

A similar interpretation can be advanced to describe the shortening of the unirradiated axoneme in Actinophrys. Since this axopodium shortens at a time when irradiated axopodia are growing out it appears most unlikely that damage to the unirradiated axopodium is responsible for its shortening. This is a clear example of a situation where some

microtubules are assembling whilst others are breaking down in the same cell.

In conclusion, it is suggested that these experiments indicate that the level of available precursor material may in certain situations limit the rate of outgrowth, and might also influence the determination of the length, of axopodia.

APPENDICES

APPENDIX I

The Determination of Young's Modulus for the Axopodium

(a) Derivation of Equation (1)

Provided that the bond between the axopodium and the glass fibre is firm enough to allow no movement, and as the perpendicular distance of the base of the glass fibre from the base of the axopodium is much greater than the lengths (x_1 and x_2) of the bent regions of the axopodium and the glass fibre respectively, then the bending moments in the axopodium and the glass fibre are equal (Fig. 15).

The bending moment in the glass fibre is the product of the force (F) produced by the movement of the base of the fibre and the distance (L) from the stiff base of the fibre to the plane of the point of attachment of the axopodium to the cell body:

i.e. bending moment = FL

The bending moment in the glass fibre can also be calculated knowing

Young's Modulus of the glass, E_2,

the moment of inertia of the glass fibre, I_2, and

the radius of the curve it is bent into, R_2,

$$\therefore FL = \frac{E_2 I_2}{R_2}$$

But the bending moment in the glass fibre equals that in the axopodium,

$$\therefore \frac{E_1 I_1}{R_1} = \frac{E_2 I_2}{R_2}$$

$\therefore$ where E_1, I_1, and R_1 are the Young's Modulus, moment of inertia and radius of curvature of the axopodium respectively,

$$E_1 = E_2 \frac{R_1}{R_2}\left(\frac{I_2}{I_1}\right) \quad . \quad . \quad . \quad . \quad . \quad (4)$$

Now where a_1 and a_2 are the radius of the axopodium and the rod respectively,

$$I_1 = \frac{\pi a_1^4}{4}, \text{ and } I_2 = \frac{\pi a_2^4}{4}$$

$\therefore$ substituting in Equation (4),

$$E_1 = E_2 \frac{R_1}{R_2}\left(\frac{a_2}{a_1}\right)^4 \quad . \quad . \quad . \quad . \quad . \quad (1)$$

(b) Determination of (E_2): Young's Modulus for the glass tube

The moment of inertia (I_2) of a tube

$$= \frac{\pi}{4}(r_2^4 - r_1^4) \quad . \quad . \quad . \quad \text{Equation (5)}$$

where r_2 = external radius of glass tube = 0.803 mm.

r_1 = radius of lumen of glass tube = 0.533 mm.

Substituting in Equation 5,

$$I_2 = 2.6 \times 10^{-5} \text{ gm cm}^{-2}$$

The glass tube was clamped at a horizontal distance x (29.08 cm) from its tip. It was loaded at its tip with a series of weights (M). The deflection (y) of the tip of the beam from the horizontal was measured at each addition of new weight. The deflection ($\dot{y}$) of the tube was also measured during unloading. Values of $\dot{y}$ (averages of the loading and unloading figures) were plotted against M.

The slope $\frac{y}{M} = \frac{gx^3}{3E_2I_2} \quad . \quad . \quad . \quad . \quad . \quad . \quad \text{Equation (5)}$

Now substituting experimentally determined values,

$$E_2 = 4.8 \times 10^{11} \pm 1.8 \times 10^{11} \text{ dyne cm}^{-2}$$

APPENDIX II

Cytochalasin B

Microscopical examination of 10-50 μgm ml^{-1} "solutions" of cytochalasin B in 1% dimethyl sulphoxide in culture medium reveals many undissolved crystals of cytochalasin B (Plate 29). Because of this and because the solutions of cytochalasin B had no detectable effect on binary fission in Actinophrys (Chapter II), it was necessary to ensure that the solutions were "biologically active". The system chosen (as a result of a suggestion by Dr H. Stebbings) was the streaming of cytoplasm in Tradescantia stamen hairs (Plate 32). Cytochalasin B inhibits this streaming (Plate 31) in a reversible and concentration-dependent fashion (Table VIII). Slight variability is seen between the effects of concentrations of solutions made up by different methods. Solutions of cytochalasin B first dissolved in 100% dimethyl sulphoxide and then diluted with culture medium begin to inhibit cytoplasmic streaming at higher "apparent concentrations" than do solutions where crystalline cytochalasin B is dispersed into already diluted dimethyl sulphoxide (Table VIII). Solutions of cytochalasin B which inhibit cytoplasmic streaming in Tradescantia do not appear to inhibit the movement of haptocysts along axopodia in Actinophrys (Table VIII; Plate 30).

TABLE VIII: The effect of cytochalasin B

	Control solutions		Cytochalasin B solutions μgm ml^{-1}					Cytochalasin B dispersions μgm ml^{-1}				
	Culture medium	1% DMSO & culture medium	0.1	1	5	10	50	0.1	1	5	10	50
Movement of haptocysts in Actinophrys	+	+	+	+	+	+	+	+	+	+	+	+
Cytoplasmic streaming in Tradescantia	+	+	+	+	±	–	–	+	–	–	–	–

Table VIII reveals that the solutions of cytochalasin B used were biologically active. The possibility (mentioned in Chapter II) that Actinophrys is impermeable to cytochalasin B remains open, because haptocyst movement was not affected. It should be noted that quoted concentrations of cytochalasin B solutions are potentially quite inaccurate and therefore useful only as a reference.

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PLATES

Unless otherwise stated all plates are transmission electron micrographs of organisms fixed using procedure 1 (Materials and Methods). Abbreviations have been used to show where other types of microscopy have been employed:

P — Zernicke phase contrast
Nom — Nomarski differential interference contrast
I — Interference microscopy
SEM — Scanning electron microscopy

PLATE 1

A living organism showing the radial arrangement of axopodia.
x 1452 (Nom)

FIGURE 6

An optical densitometer tracing obtained by scanning across an electron micrograph of a portion of the nuclear envelope sectioned perpendicularly to the plane of the envelope. The absorbance is plotted in arbitrary units and the peaks represent regions of greater electron density. The outer and inner peaks represent the nuclear envelope and the two layers of enclosed dense material, respectively. The abscissa indicates the length of the scan in terms of the spacing of structures in the section.

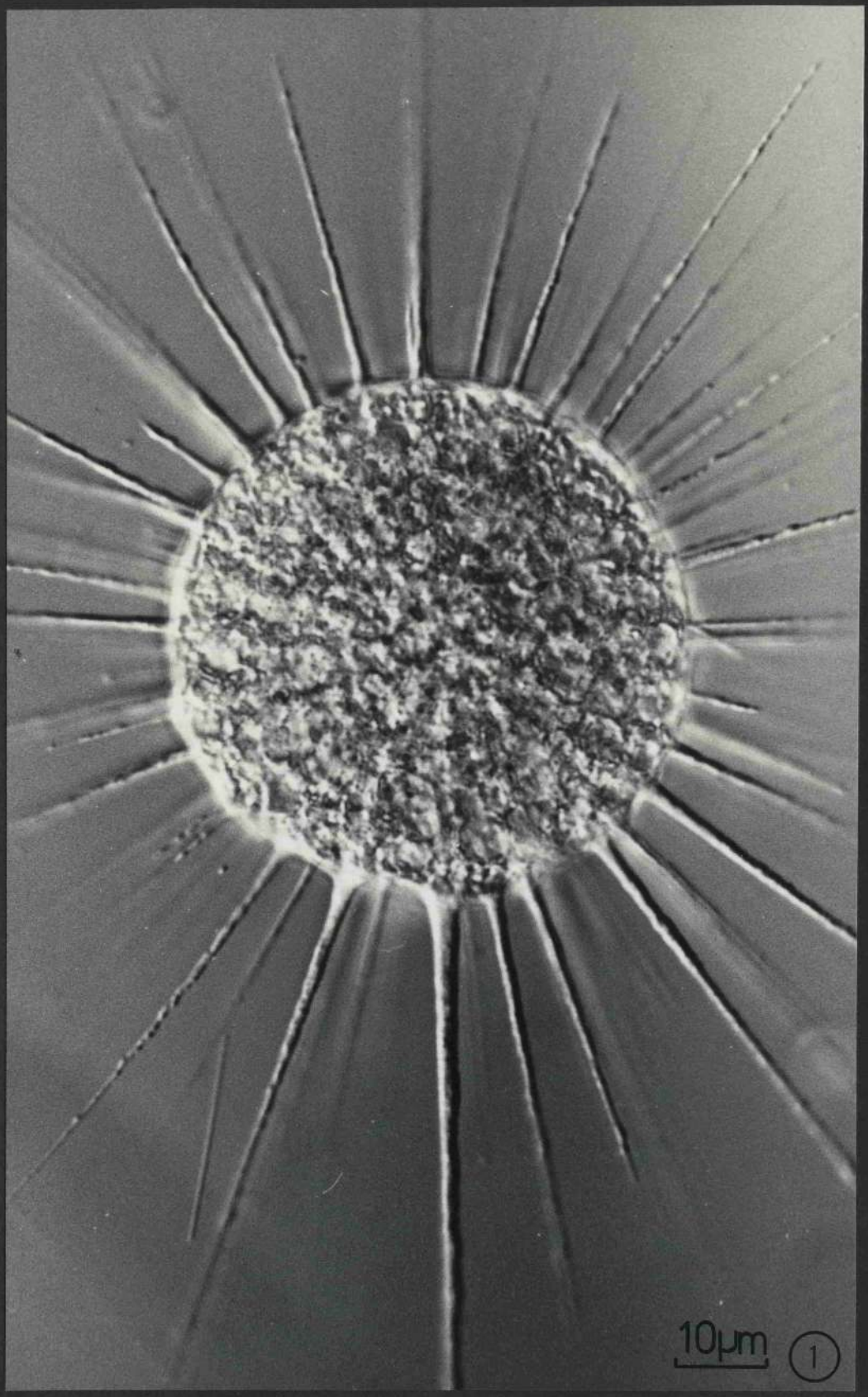
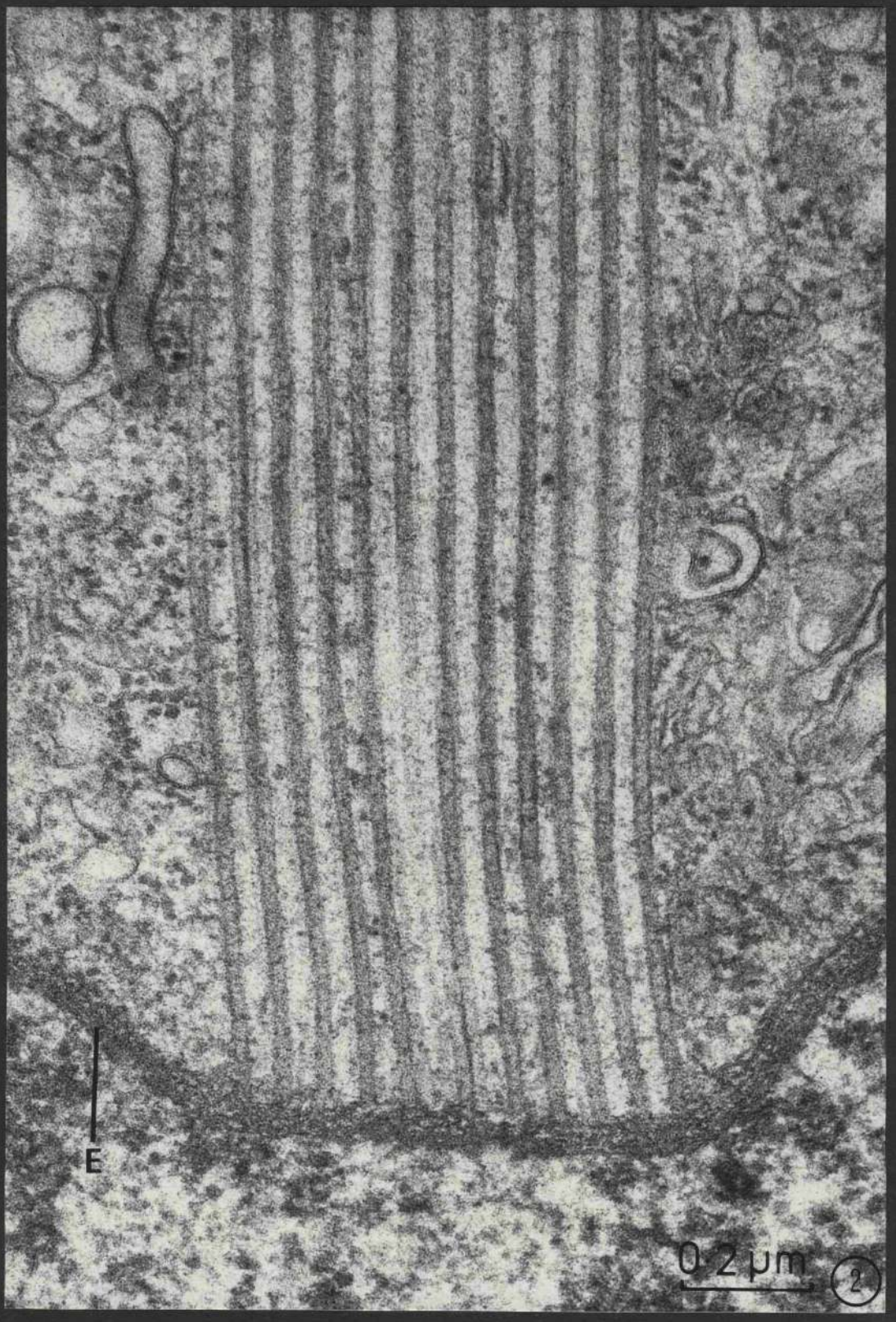

10µm
1

PLATE 2

The basé of a large axoneme, sectioned longitudinally, contacting the nuclear envelope (E).
x 107,692

PLATE 3

A portion of the nuclear envelope sectioned perpendicularly to the plane of the envelope. Two layers of dense material (D) are situated inside the envelope (E) and three pores (P) are also shown. The cytoplasmic surface of the envelope is towards the bottom of the figure. Fixation procedure 2.
x 160,000


E
0·2 µm
2

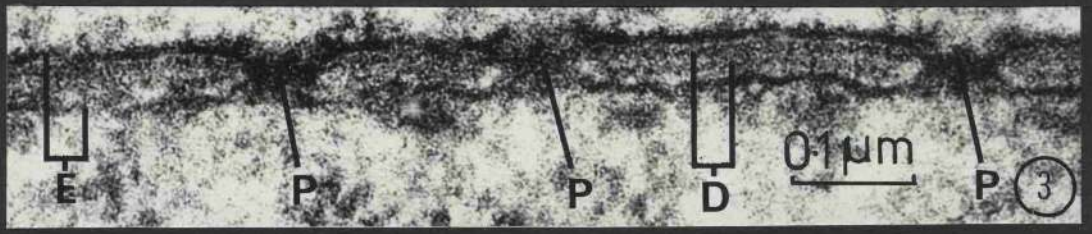

E
P
P
D
0·1 µm
P
3

PLATE 4

A cross-section of a large axoneme within the cell body. Long links (L) extend between tubules in adjacent rows. Short links (l) occur between adjacent tubules in the same row.
x 98,181

PLATE 5

A cross-section of a large axoneme within the cell body. Several C-microtubules (arrows) are present. Fixation procedure 2.
x 97,777

PLATE 6

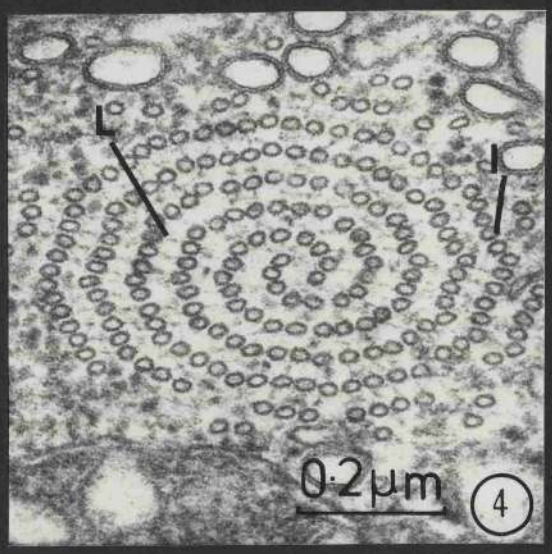
A cross-section of an axopodium. Haptocysts (H) lie adjacent to the regularly packed microtubules of the axoneme.
x 48,333

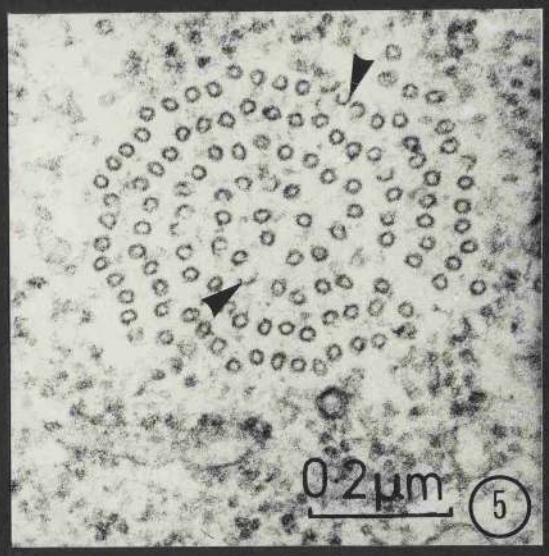
PLATE 7

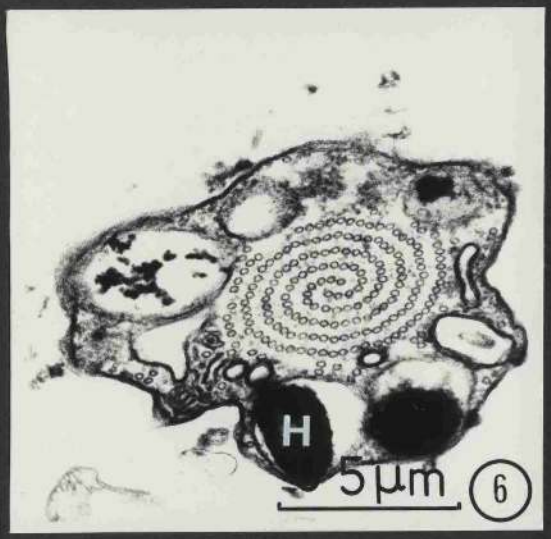
Cross-section of an axopodium with irregularly packed microtubules. A haptocyst (H) with dense contents is also shown.
x 55,000

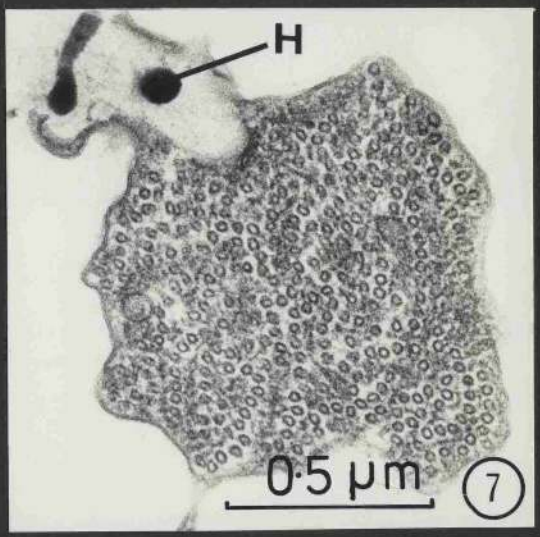
PLATE 8

Longitudinal section of part of a large axoneme showing the "stepped" configuration at its base which contacts the nuclear envelope (E).
x 82,857


L
I
0·2μm
4


0·2μm
5


H
5μm
6


H
0·5 μm
7

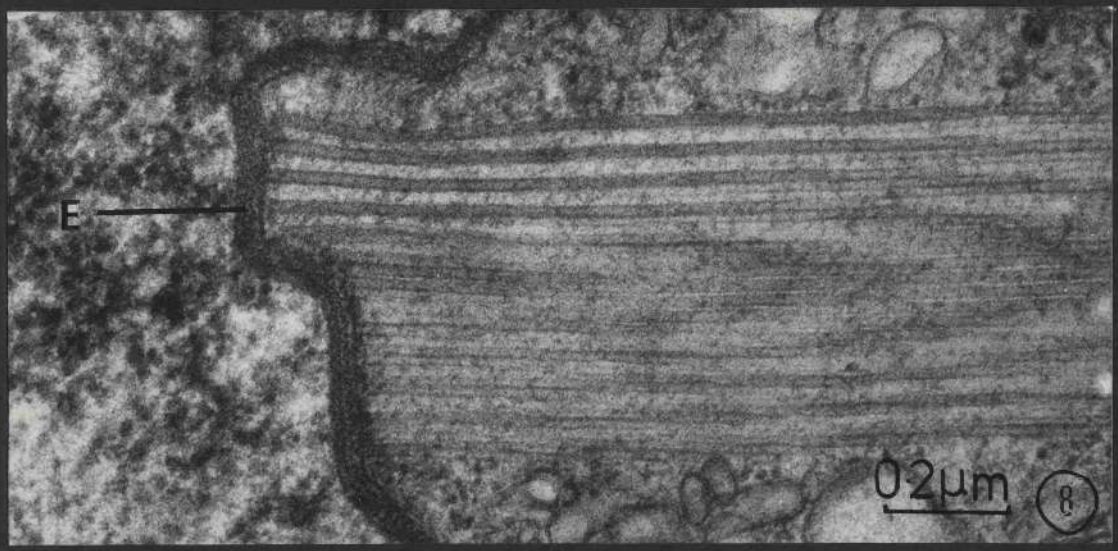

E
0·2μm
8

PLATE 9

Section of part of an encysted organism. The outer layer (O), the intermediate zone (IZ), the zygote walls (Z) and part of the cytoplasmic region (C) are shown. The cytoplasmic region contains cytoplasmic spheres (CS).
x 8,461

PLATE 10

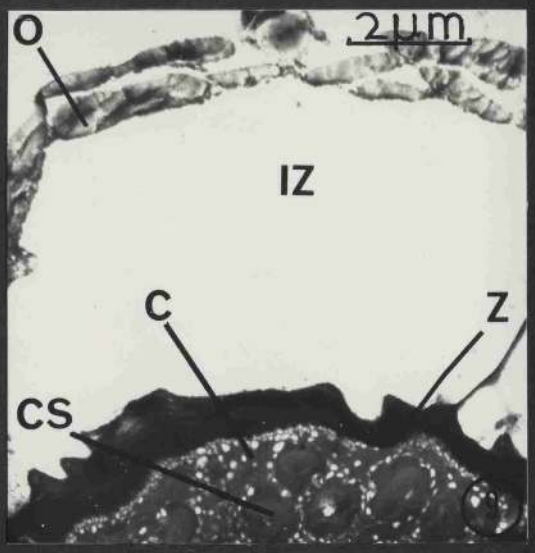
The surface of part of the outer layer of an encysted organism.
x 9,090 (SEM)

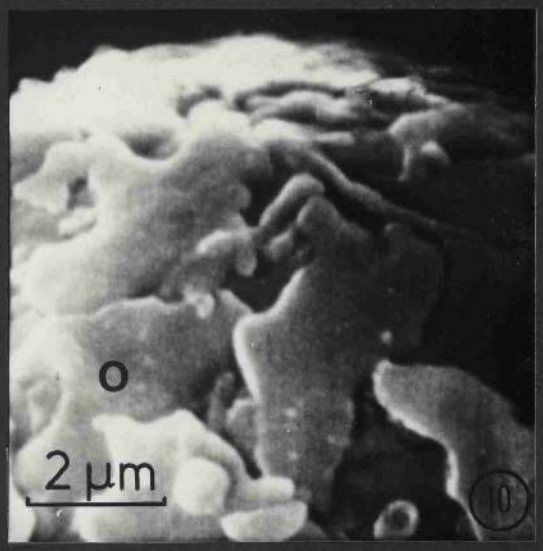
PLATE 11

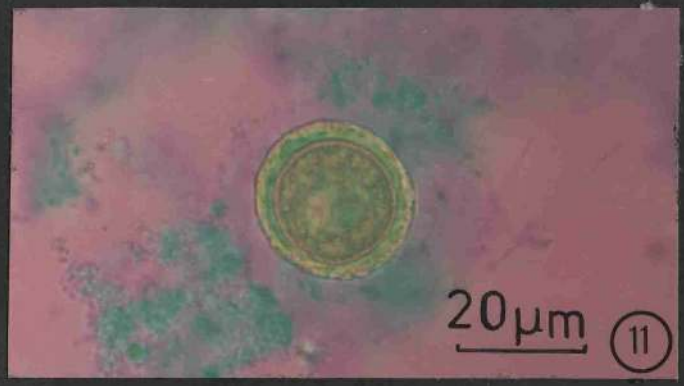
Whole encysted organism. The yellow outer layer, green intermediate zone and red zygote walls surround the cytoplasmic region (C).
x 880 (I)

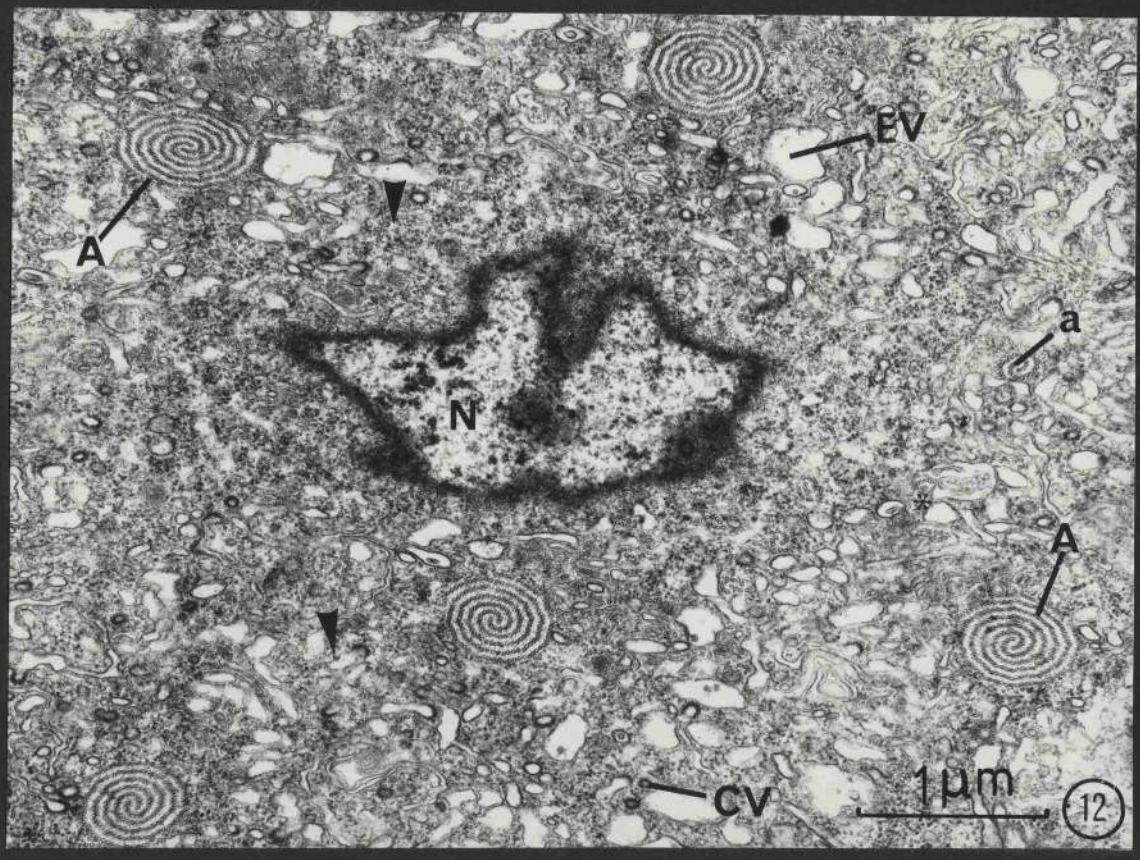
PLATE 12

A large area of endoplasm is seen in this section which "grazes" the edge of the nucleus (N). Several large axonemes (A) do not have the same axis of symmetry. A few small axonemes (a) and single tubules (arrows) occur. Electron-lucent endoplasmic vesicles (EV) and coated vesicles (CV) occur throughout the endoplasm. Occasionally small single rows of microtubules occur (asterisk).
x 25,000


O
2µm
IZ
C
Z
CS


O
2 µm
10


20µm
11

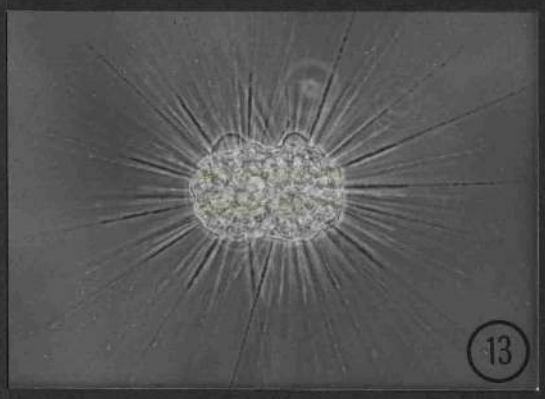

EV
A
a
N
A
CV
1µm
12

PLATES 13-20

Show stages in binary fission. The bridge of cytoplasm which connects daughter cells attenuates as division proceeds. A vesicle (arrows) occludes the bridge of cytoplasm shown in Plates 15-17. The time elapsed from the start of observation is as follows:

Plate 13: 0 min
" 14: 17 "
" 15: 18 "
" 16: 18.5 "
" 17: 19 "
" 18: 20 "
" 19: 20.5 "
" 20: 23 "

x 216 (P)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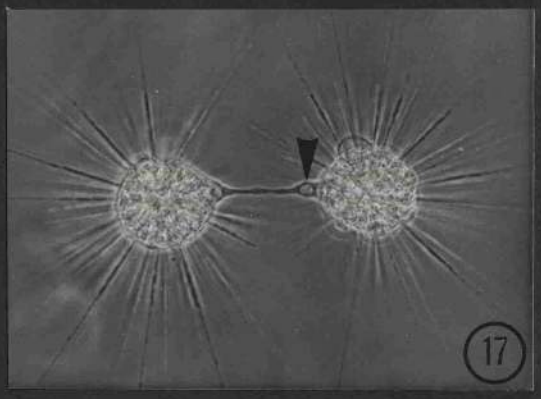
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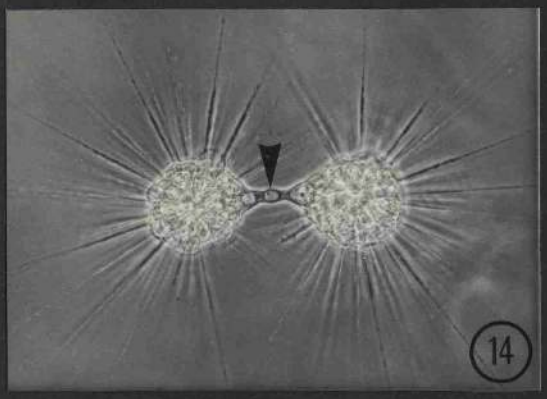
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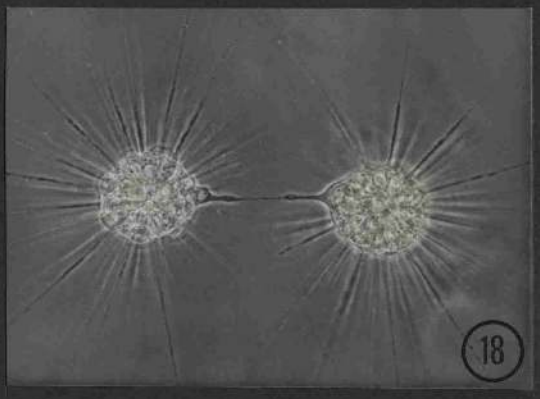
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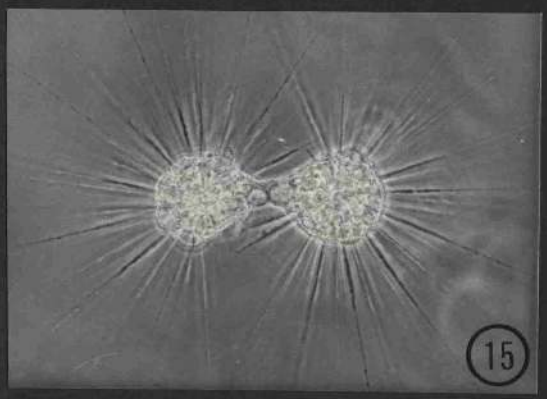
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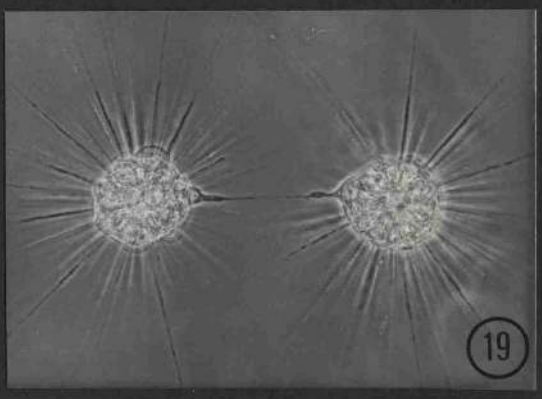
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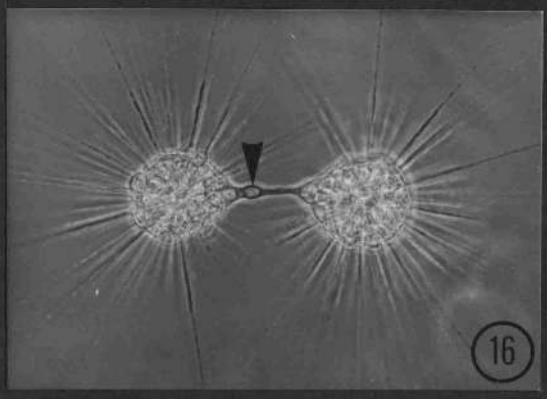
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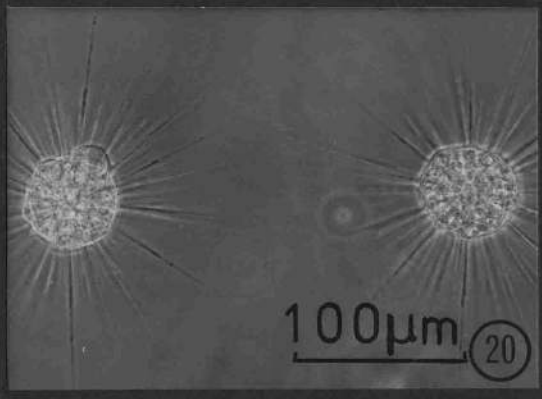

100µm
20

PLATE 21

In this dividing organism axopodia based on opposite cell bodies make double non-terminal contact with each other near their tips. A webbed region (W) can be seen at the point of contact.
x 416 (P)

PLATE 22

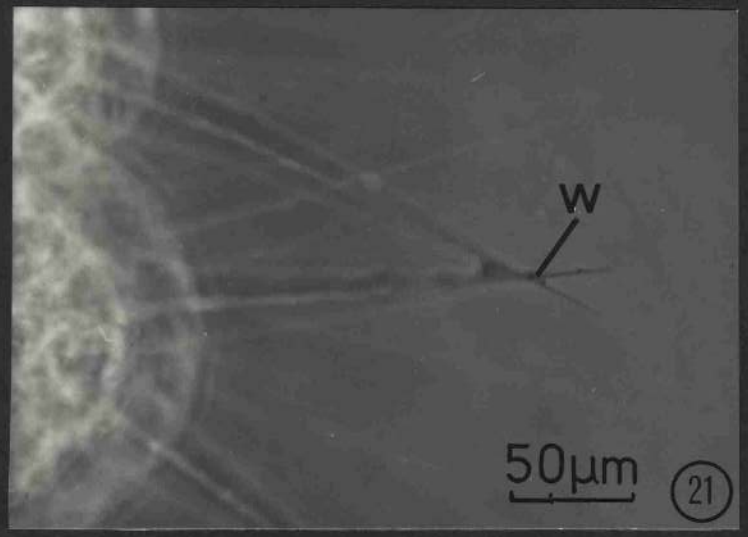
Small growing axopodia (arrows) are seen on regions of the cell bodies adjacent to the connecting bridge of cytoplasm in this dividing cell.
x 566 (P)

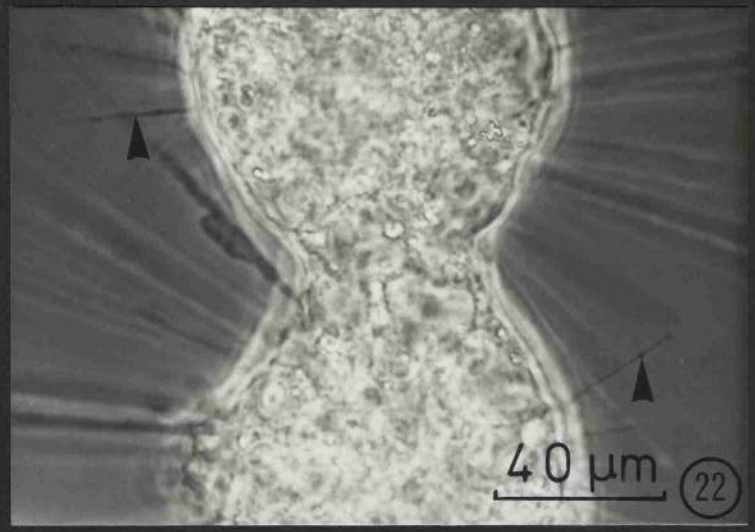
PLATE 23

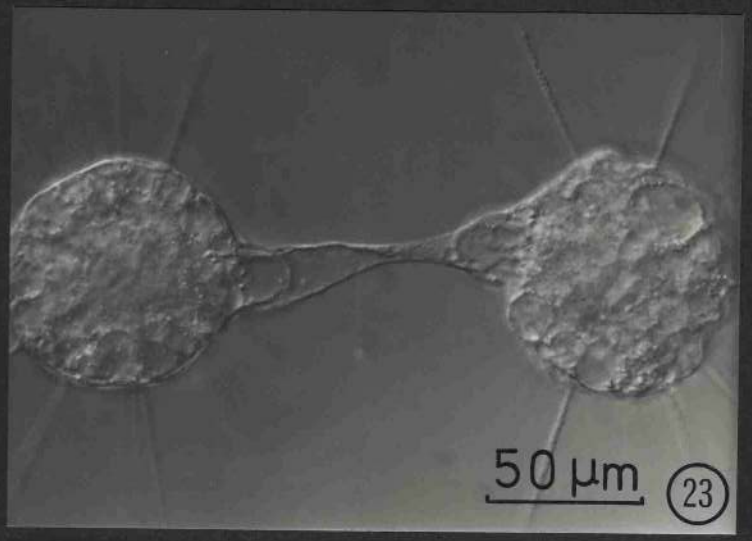
The cytoplasmic bridge connecting these dividing organisms is highly vacuolated.
x 308 (Nom)

PLATE 24

Two axopodia (arrows) based on one cell body make single terminal contacts with axopodia based on the other cell body. One of these axopodia (long arrow) is bent. Dividing organisms.
x 520 (P)


w
50µm
21


40 µm
22


50 µm
23

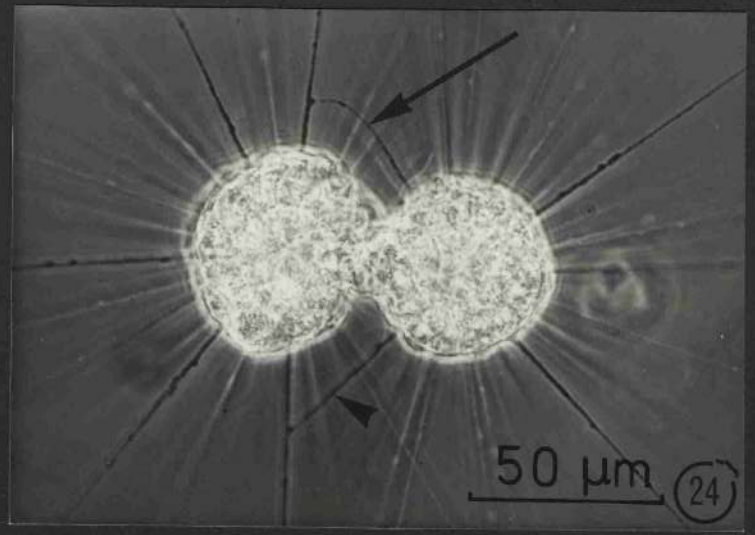
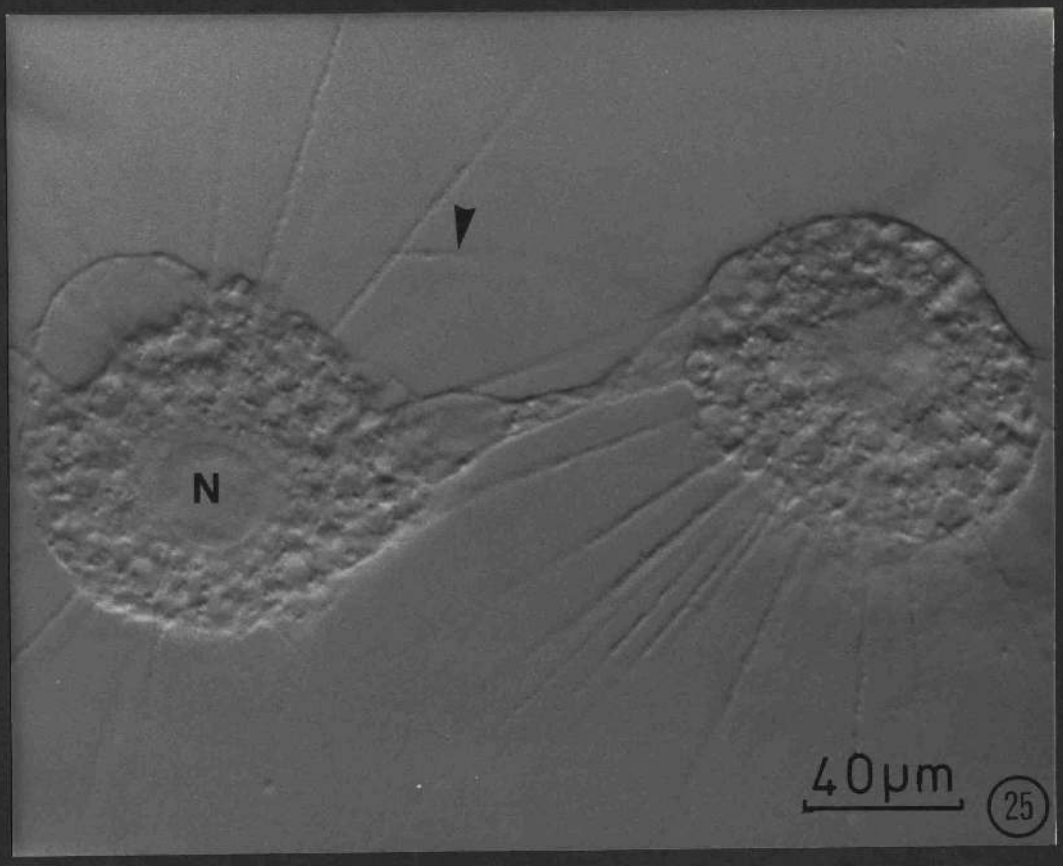

50 µm
24

PLATE 25

One of the nuclei (N) present in these dividing organisms can be seen. The tip of a bent axopodium (arrow) on one cell body makes a single terminal contact with an axopodium on the other cell body.
x 536 (Nom)

PLATE 26

A group of Actinophrys in the colonial state. One organism (bottom right) is shown in the process of splitting off from the clump. A bent axopodium (arrow) can be seen in the region of the connecting bridge of cytoplasm.
x 345 (Nom)


N
40µm
25

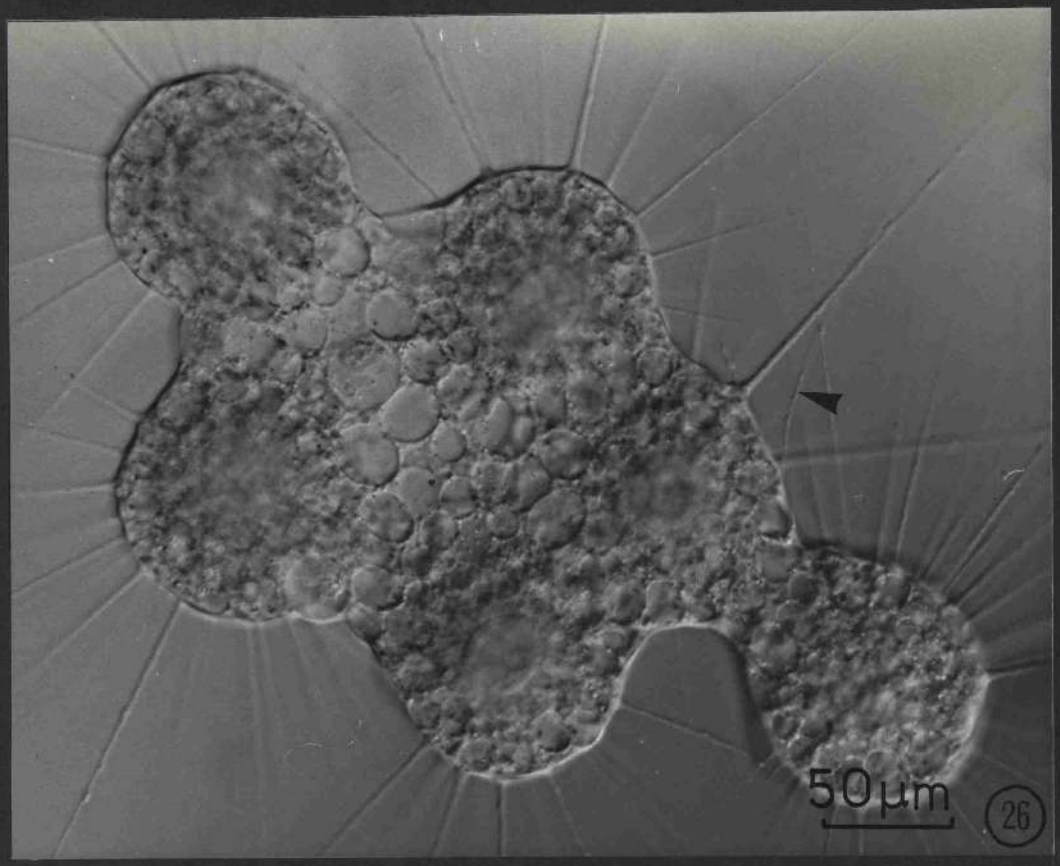

50µm
26

PLATE 27

A transverse section of the bridge of cytoplasm connecting two dividing daughter cells. Examination of sections of this type revealed neither microtubules nor microfilaments.
x 2,310

PLATE 28

A longitudinal section of the bridge of cytoplasm connecting two dividing daughter cells. The arrangement of vacuoles in the bridge is such that no long straight skeletal structure could pass from one cell body to the other.
x 2,555

PLATE 29

Crystals (cr) are visible when "solutions" with a concentration of cytochalasin B above 10^{μ}gm/ml are examined microscopically.
x 170 (P)

PLATE 30

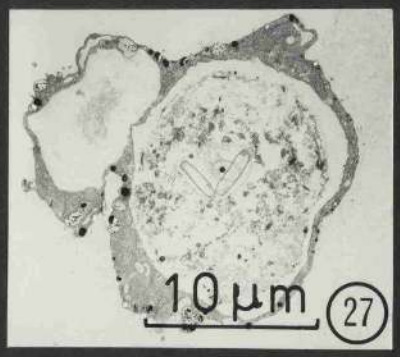
An organism treated with 50^{μ}gm/ml of cytochalasin B. The axopodia do not retract, and the movement of haptocysts along axopodia is not inhibited.
x 353 (Nom)

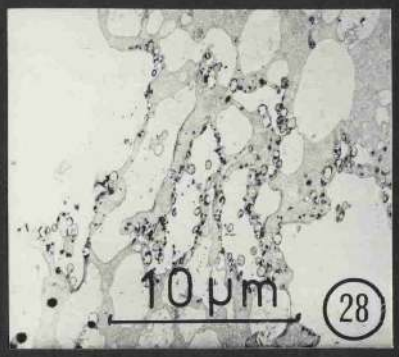
PLATE 31

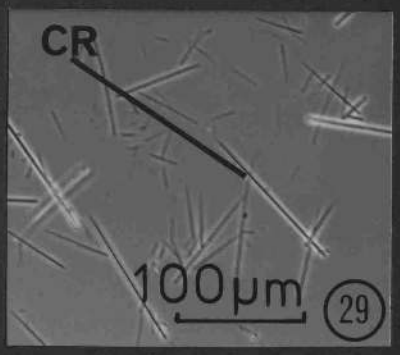
After treatment with 10^{μ}gm/ml cytochalasin B the cytoplasmic streaming in this stamen hair cell from Tradescantia has ceased. The strands of cytoplasm have withdrawn and surround the nucleus (N).
x 423 (Nom)

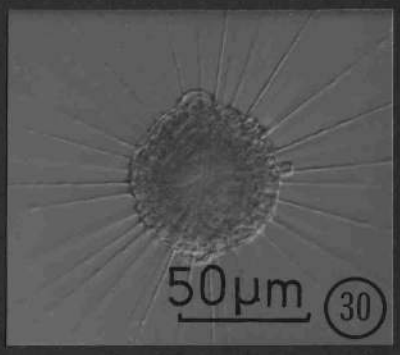
PLATE 32

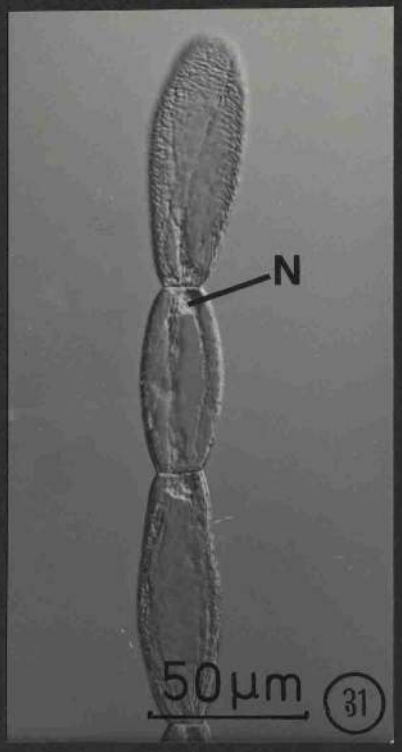
The tip of an untreated staminal hair from a Tradescantia flower. In each cell strands of actively streaming cytoplasm (arrows) radiate from the nucleus (N).
x 246 (Nom)


10µm
27


10µm
28


CR
100µm
29


50µm
30


N
50µm
31

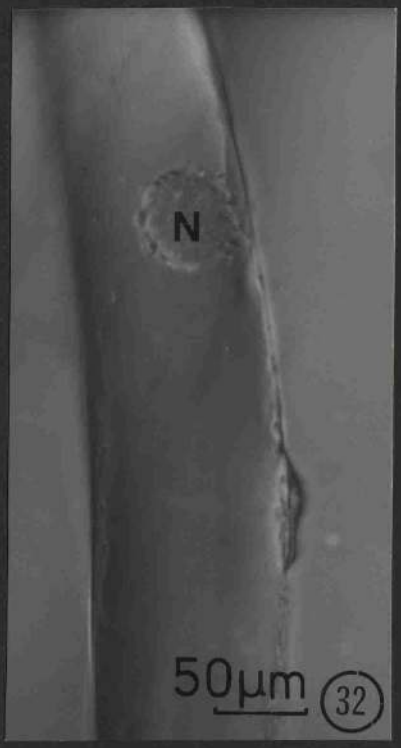
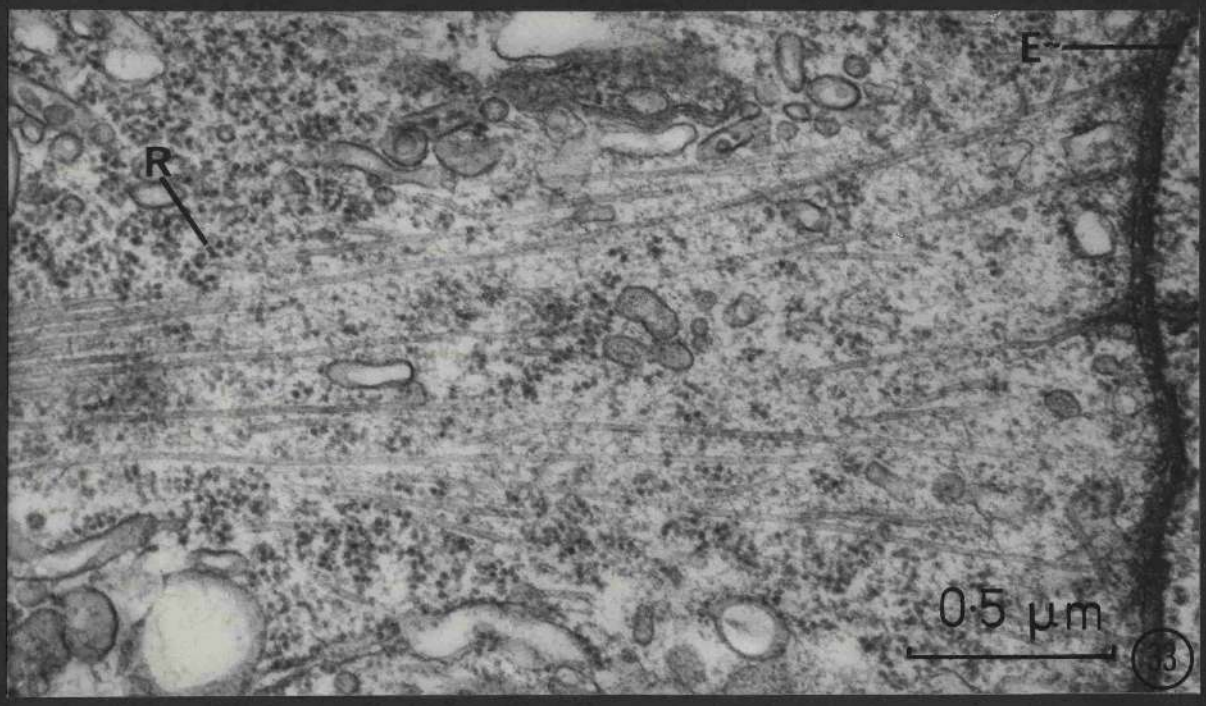

N
50µm
32

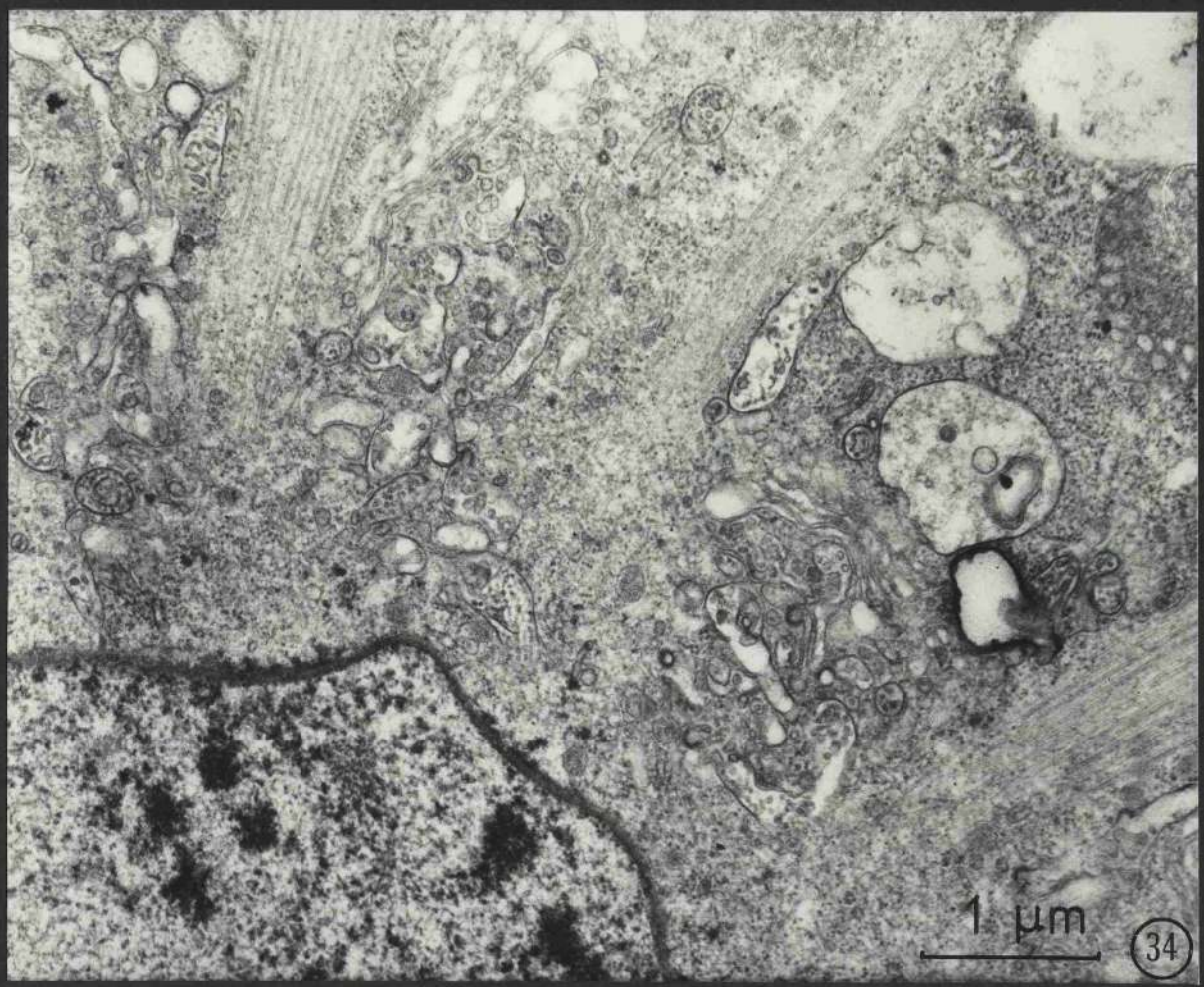
PLATE 33

An organism fixed in a late stage of division (later than the stage shown in Plate 14) has axonemes of unusual morphology. Longitudinal sections of this axoneme show its "splayed" appearance where the tubules at its base contact the nuclear envelope. Such axonemes may be in the process of reattaching to the nuclear envelope (E). Polyribosomes can be seen in this region.
x 54,000

PLATE 34

A section through an organism fixed at an earlier stage of division than that shown in Plate 14 reveals three axonemes which do not contact the nuclear envelope at their bases.
x 24,516


E
R
0·5 µm
33


1 µm
34

PLATES 35-37

Show stages in the distally directed fusion of two axopodia joining at the points arrowed until (Plate 37) they are united at their tips to form a single axopodium.
x 700 (P)

PLATE 38

Cross-section of an axopodium containing two axonemes.
x 46,857

PLATE 39

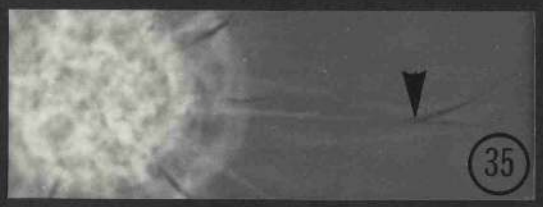
A bend (arrow) is moving towards the tip of an axopodium (Ap).
x 360 (P)

PLATE 40

The same axopodium photographed 2 min later; the bend has just been repaired. Each swollen portion (H) is a region where haptocysts lie between the axoneme and the cell membrane.
x 360 (P)

PLATE 41

The apparatus used for micromanipulation experiments. Movements of the joystick (J) are transmitted hydraulically to the glass microneedles. For the purposes of this illustration the condenser (K) of the inverted microscope was raised above its normal operational position.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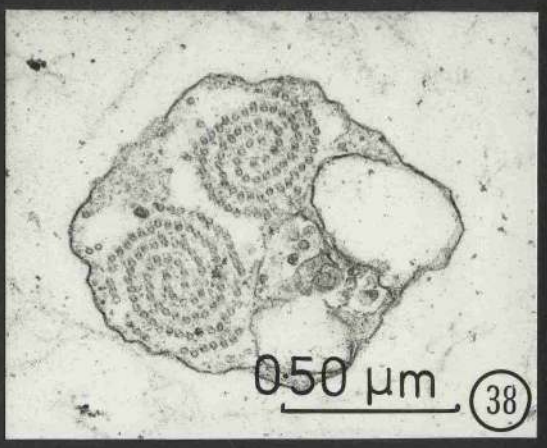
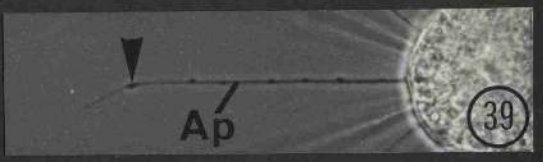

050 µm
38


36


Ap
39


30 µm
37


H
60µm
40

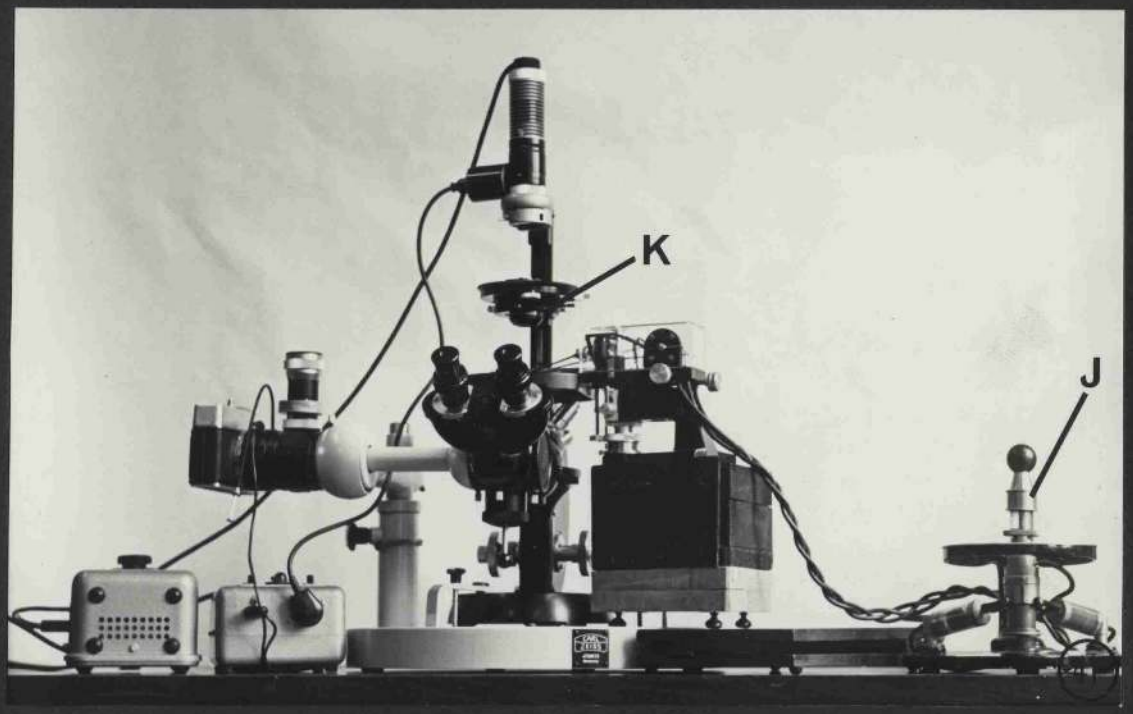

K
J

PLATE 42

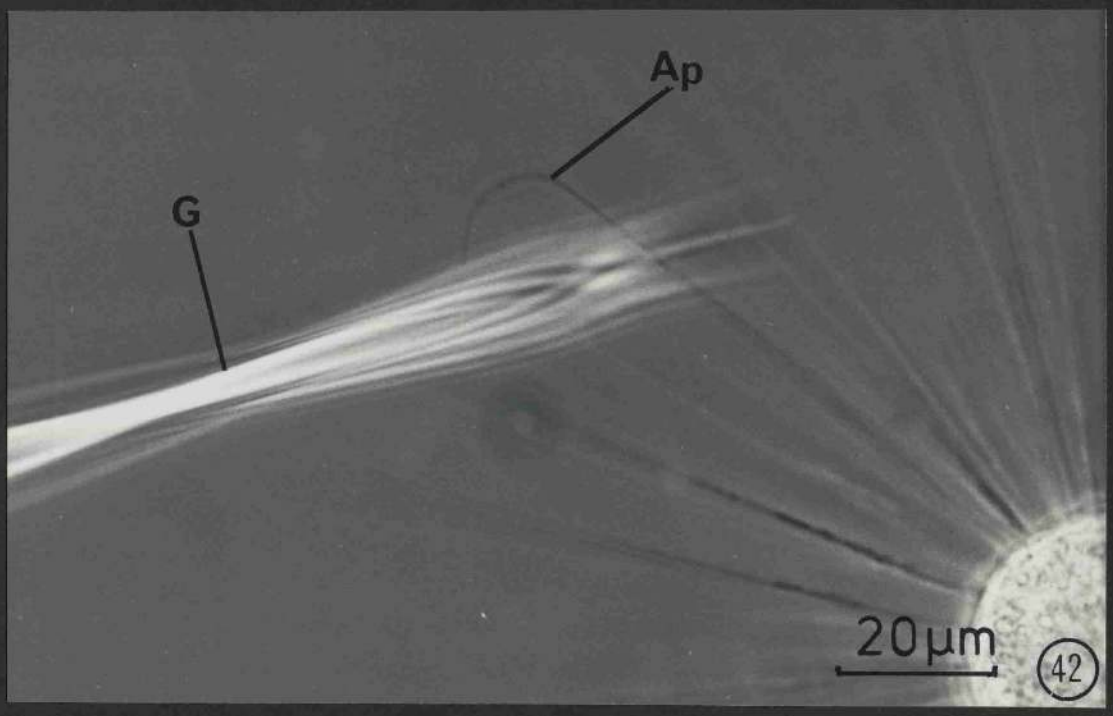
The tip of the axopodium (Ap) has stuck to the side of the glass needle (G) which has been used to bend the axopodium.
x 1,073 (P)

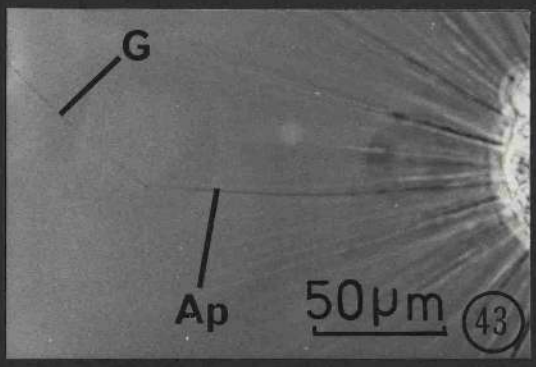
PLATE 43

The fine glass needle (G) and the axopodium (Ap) are both bent. This photograph has been used to determine Young's Modulus for the axopodium depicted.
x 352 (P)

PLATE 44

Severe manipulations with a stiff glass needle (G) have caused this axopodium (Ap) to break.
x 1,222 (P)


Ap
G
20µm
42


G
Ap
50µm
43

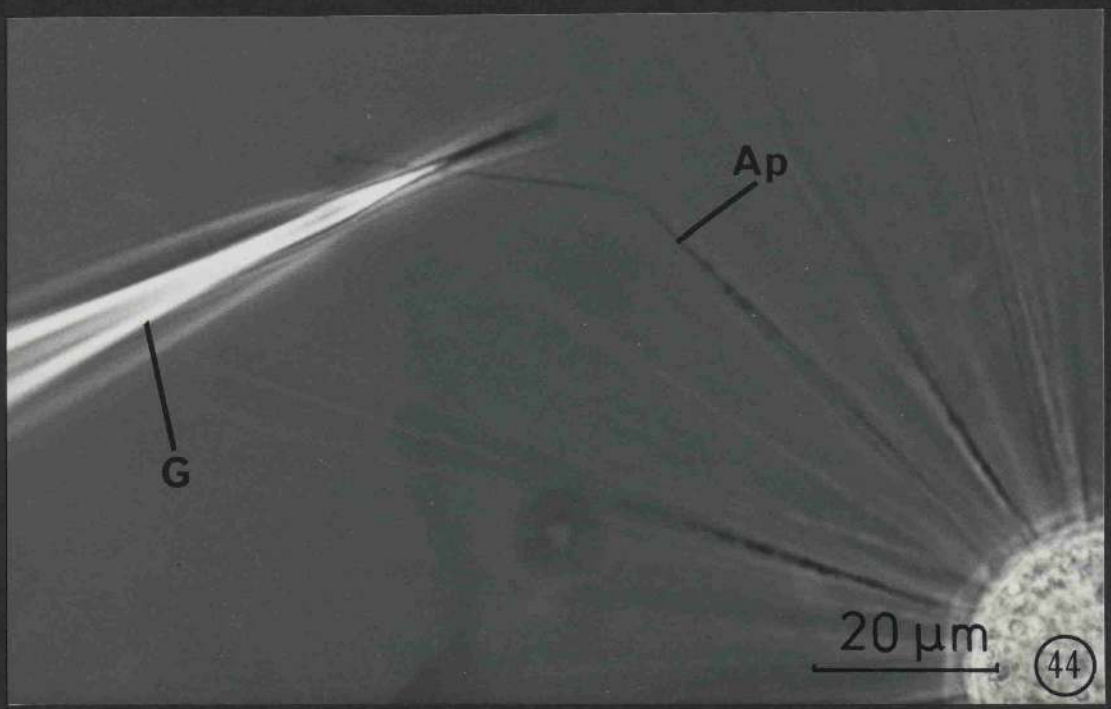

Ap
G
20 µm
44

PLATE 45

An axopodium which has just contracted rapidly has a swollen tip (T). It has drawn a flagellate (F), which is not in the same focal plane, towards the cell body (B).
x 1,470 (P)

PLATE 46

A flagellate (F) is being engulfed by a food-cup at the tip of a short pseudopodium.
x 680 (Nom)

PLATE 47

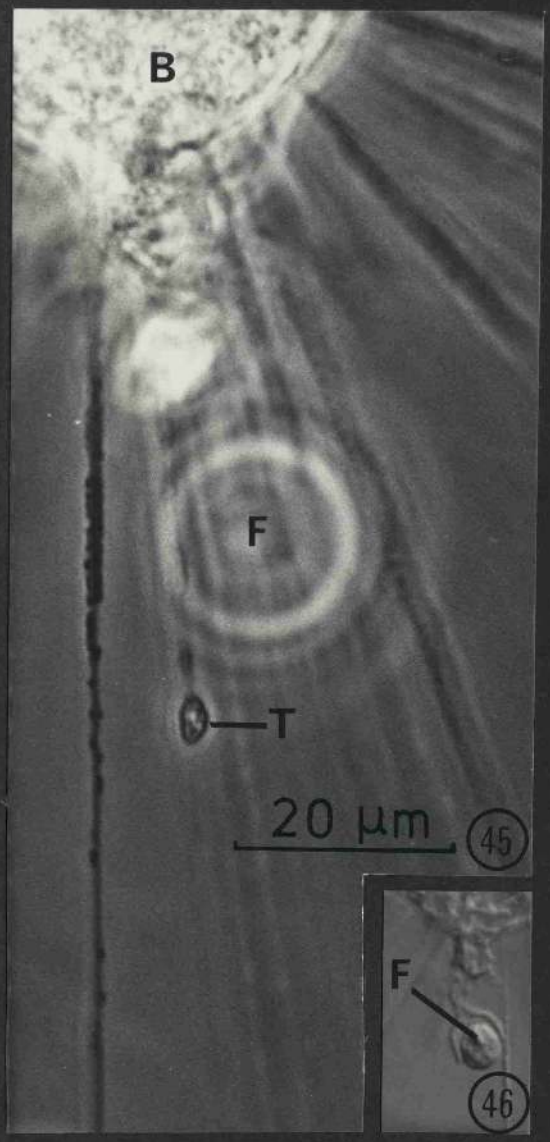
A food-cup is in the process of engulfing a captured ciliate (Tetrahymena) which has been transported to the cell body.
x 400 (P)

PLATE 48

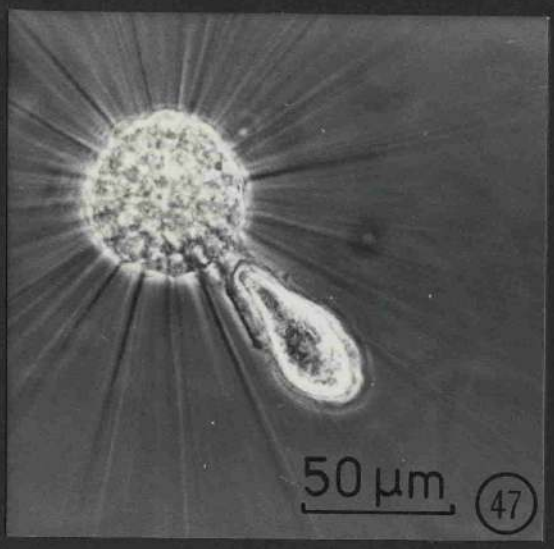
This organism has captured and engulfed a Sephadex bead (S) which has haemoglobin adsorbed to its surface. The cell surface is occasionally raised into small club-shaped projections (arrows).
x 392 (Nom)

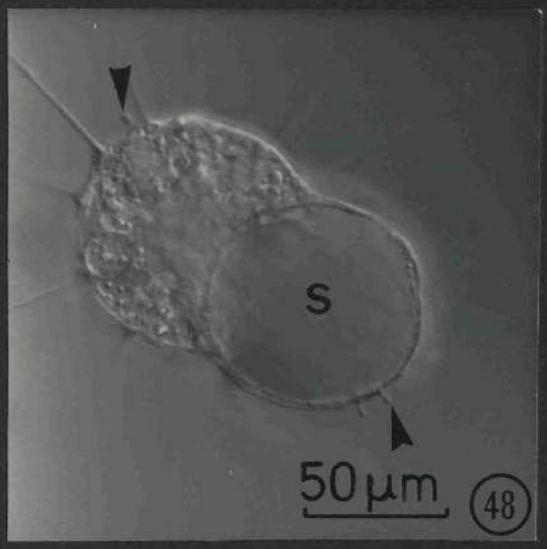
PLATES 49 and 50

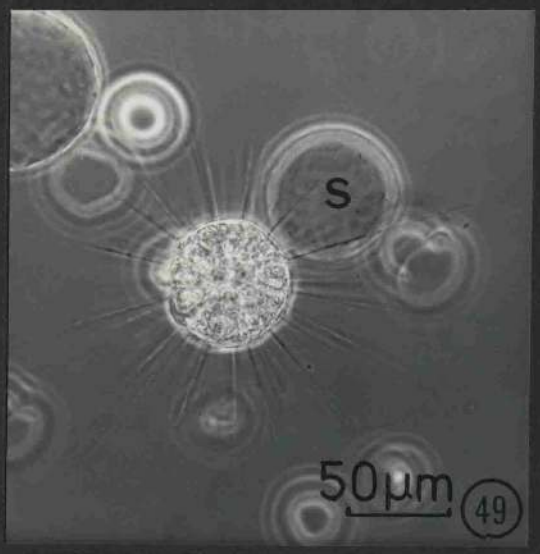
Show stages in the transport of the same Sephadex bead (S) towards the cell body (B). An axopodium (arrow) is bent out of its usual radial position as the sephadex bead is transported nearer to the cell body.
x 260 (P)

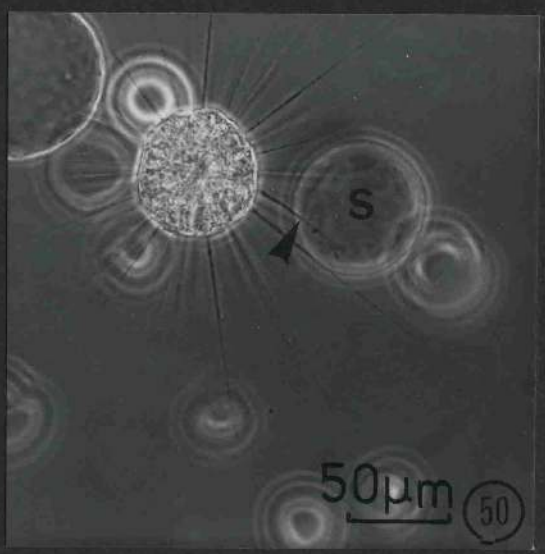

B
F
T
20 µm
45

F
46


50 µm
47


S
50µm
48


S
50µm
49


S
50µm
50

PLATES 51-53

Show successive stages in the outgrowth of an axopodium immediately after it has contracted rapidly and drawn a flagellate towards the cell body.
x 1,105 (P)

PLATE 54

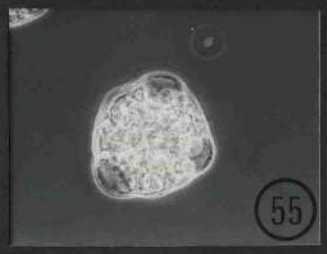
A living organism immediately after treatment at 0°C for 45 min. A few short axopodia (Ap) are still present. The central nucleus (N) is also shown.
x 720 (Nom)

PLATES 55-60

Show the recovery of an organism from cold treatment (6 hr at -3°C). Initially there appear to be no axopodia present, and the cell body is not spherical. As recovery proceeds axopodia grow out gradually becoming better radially oriented. Plate 60 shows a late stage in recovery where long radially oriented axopodia are borne on a spherical cell body. The photographs were taken at the following intervals after removal from cold:

Plate 55:	1 min
Plate 56:	2 "
Plate 57:	5 "
Plate 58:	6 "
Plate 59:	10 "
Plate 60:	45 "

x 266 (P)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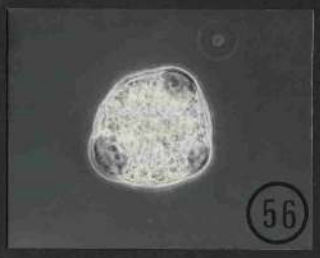
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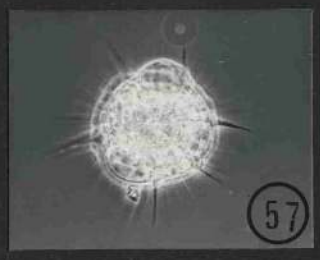
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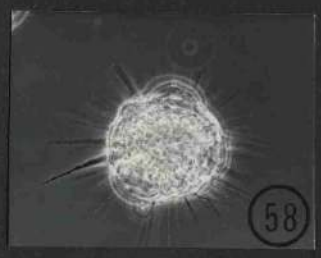
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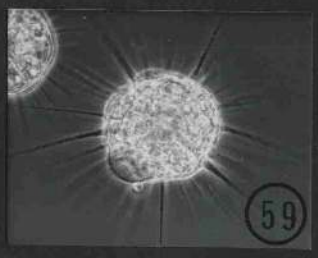
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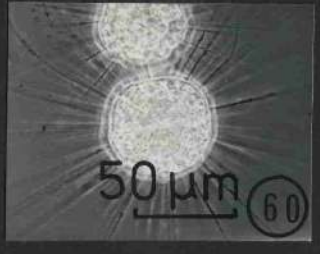
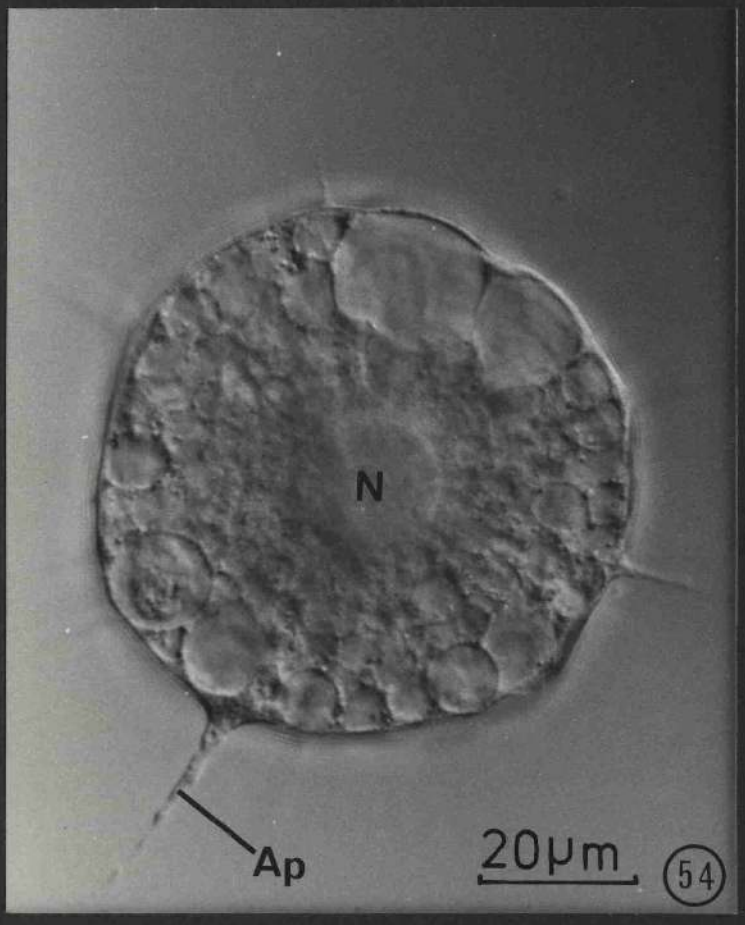

50 µm
60


51


52


20µm
53


N
Ap
20µm
54

Plates 61-64 are micrographs of sections taken from organisms treated at -3°C for 6 hr.

PLATE 61

A survey micrograph which shows a longitudinal section of one of the few axonemes (arrow) which remain after this treatment.
x 4,571

PLATE 62

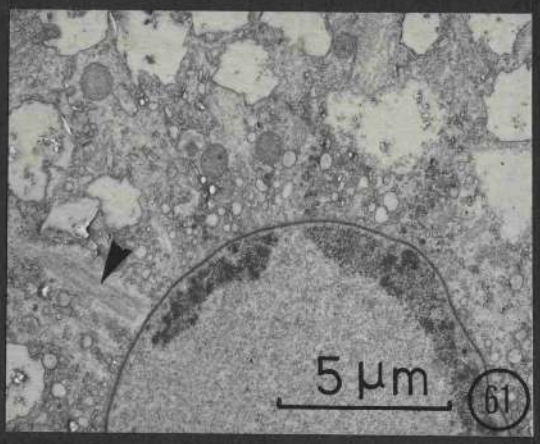
This section through the ectoplasm shows an axoneme remnant in transverse section. The axonemal microtubules are not arranged in the usual precise pattern. Many tubules appear incomplete and the circular profile is frequently indistinct.
x 114,285

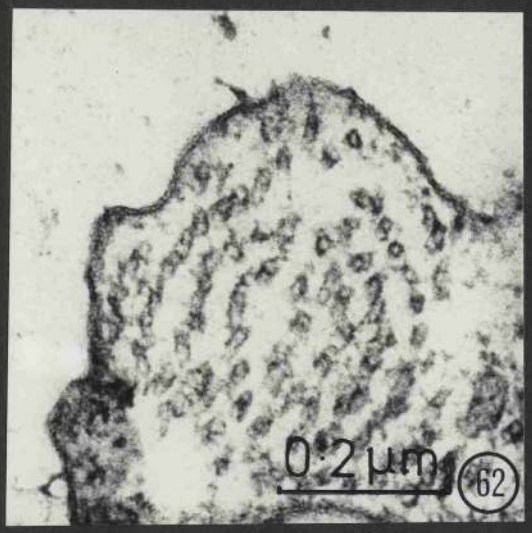
PLATE 63

Axonemal vesicles (V) occur close to these tubule remnants, situated at the border of the endoplasm and ectoplasm, seen here in longitudinal section.
x 36,666

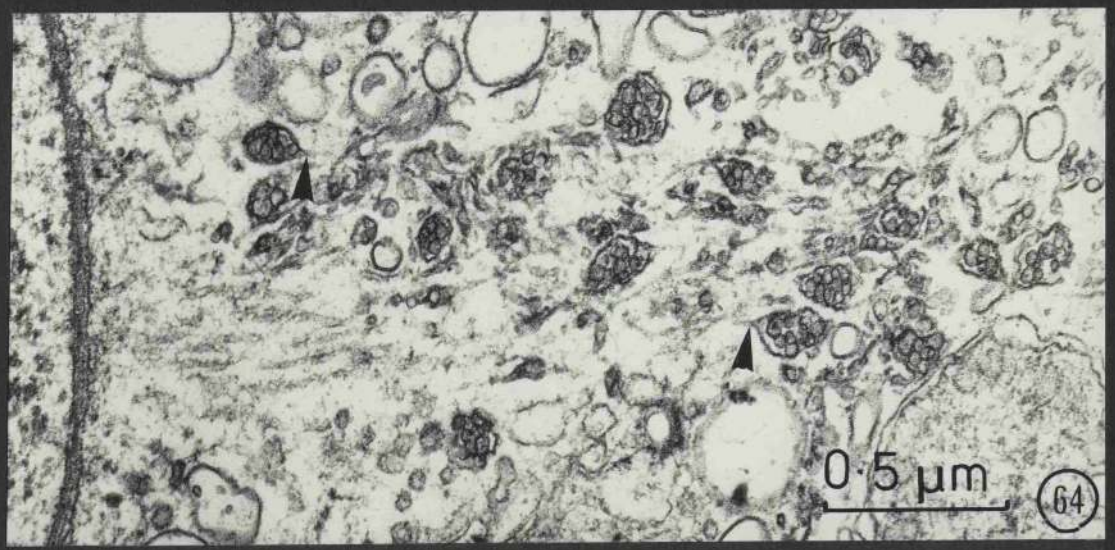
PLATE 64

In several places axonemal vesicles appear to be continuous with tubule remnants (arrows) in this longitudinal section of an axonemal remnant which lies adjacent to the nuclear envelope.
x 48,372


5 µm
61


0·2 µm
62


V
0·5 µm
63


0·5 µm
64

Plates 65-67 are micrographs of sections taken from organisms treated at -3°C for 6 hr and allowed to recover at room temperature for 1 min prior to fixation.

PLATE 65

Short lengths of microtubules are very common near to the nuclear envelope (E) which is sectioned perpendicular to its surface. Tubules appear to be oriented randomly.
x 43,333

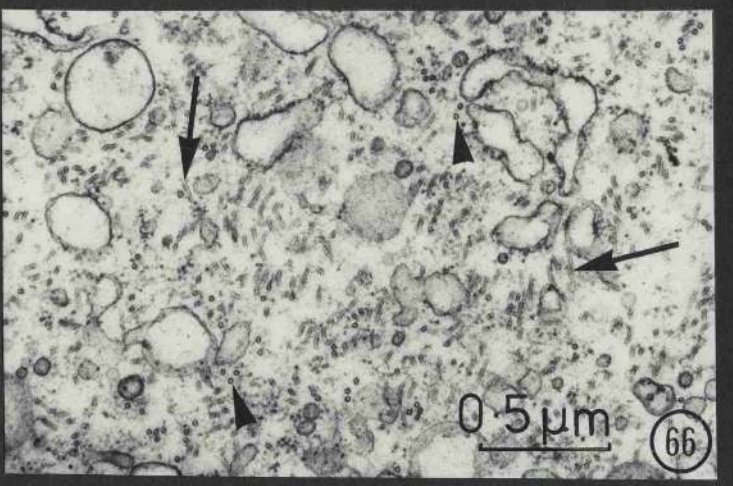
PLATE 66

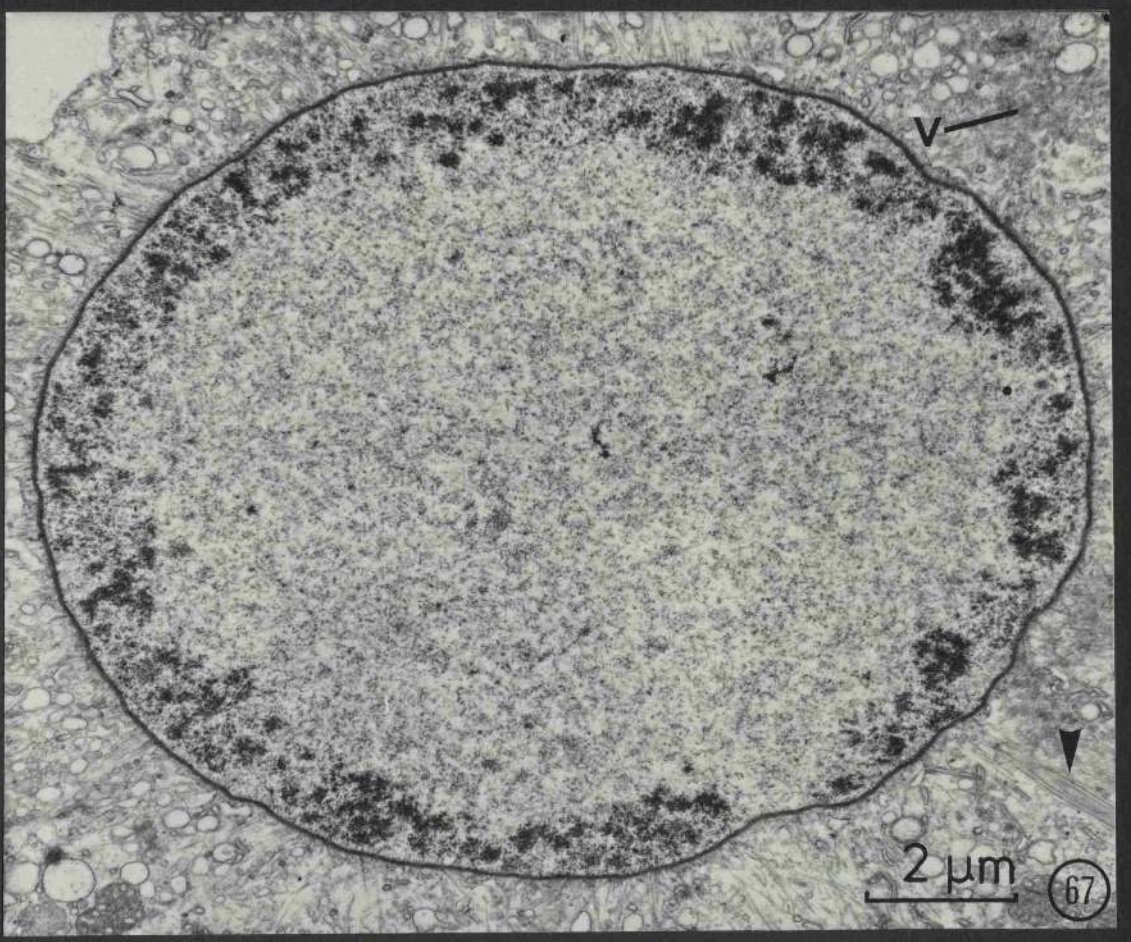
This section through the endoplasm near to the nucleus shows both the circular profiles of transverse sections through microtubules (short arrows) and short length of tubule in longitudinal section (long arrows).
x 35,882

PLATE 67

Microtubules are distributed over most of the nuclear envelope of this organism. Points of tubule attachment to the outer surface of the nuclear envelope are not grouped together.
Axonemes (arrow) occur rarely. Clusters of axonemal vesicles (V) are seen throughout the endoplasm.
x 10,901


E
0·5 µm
65


0·5 µm
66


V
2 µm
67

Plates 68-70 are micrographs of sections taken from organisms treated at -3°C for 6 hr and allowed to recover at room temperature for 2 min prior to fixation.

PLATE 68

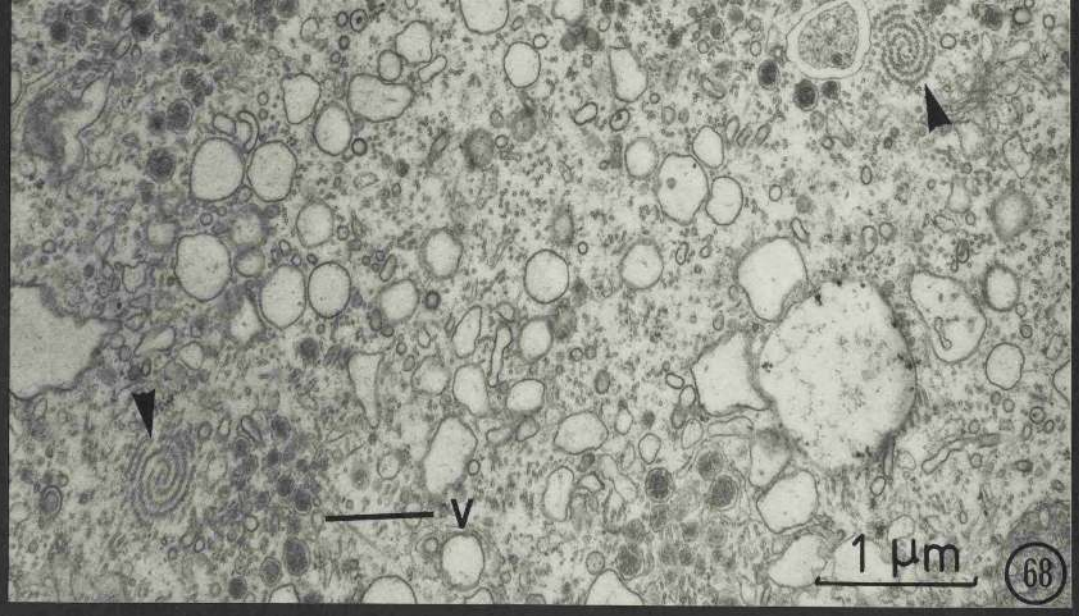
This section through the endoplasm shows two axoneme remnants in transverse section (arrows). Many large clusters of axonemal vesicles (V) are apparent. Some of these lie close to the axoneme remnants. Single tubules occur commonly in the endoplasm; they are not all oriented radially.
x 21,529

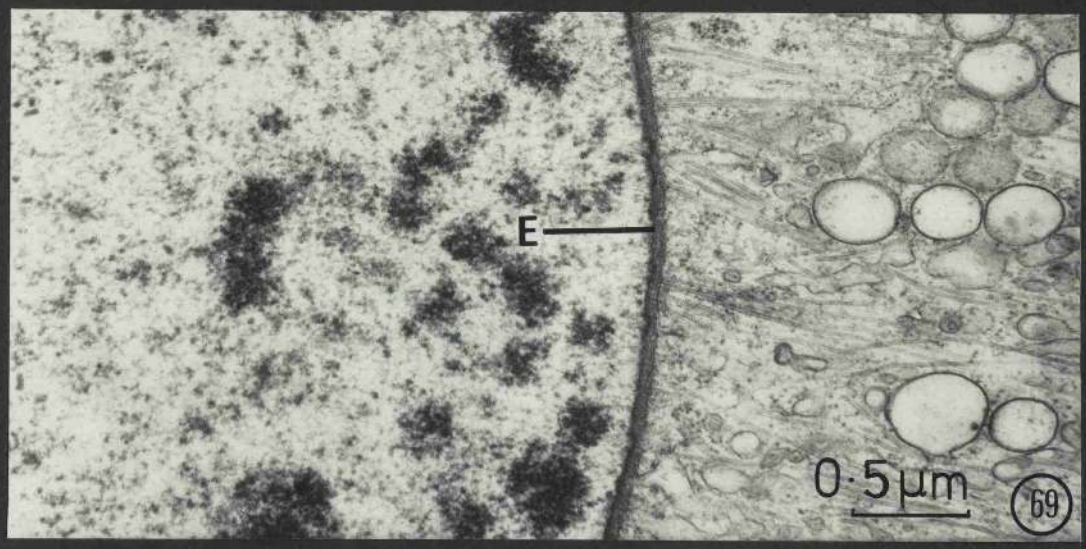
PLATE 69

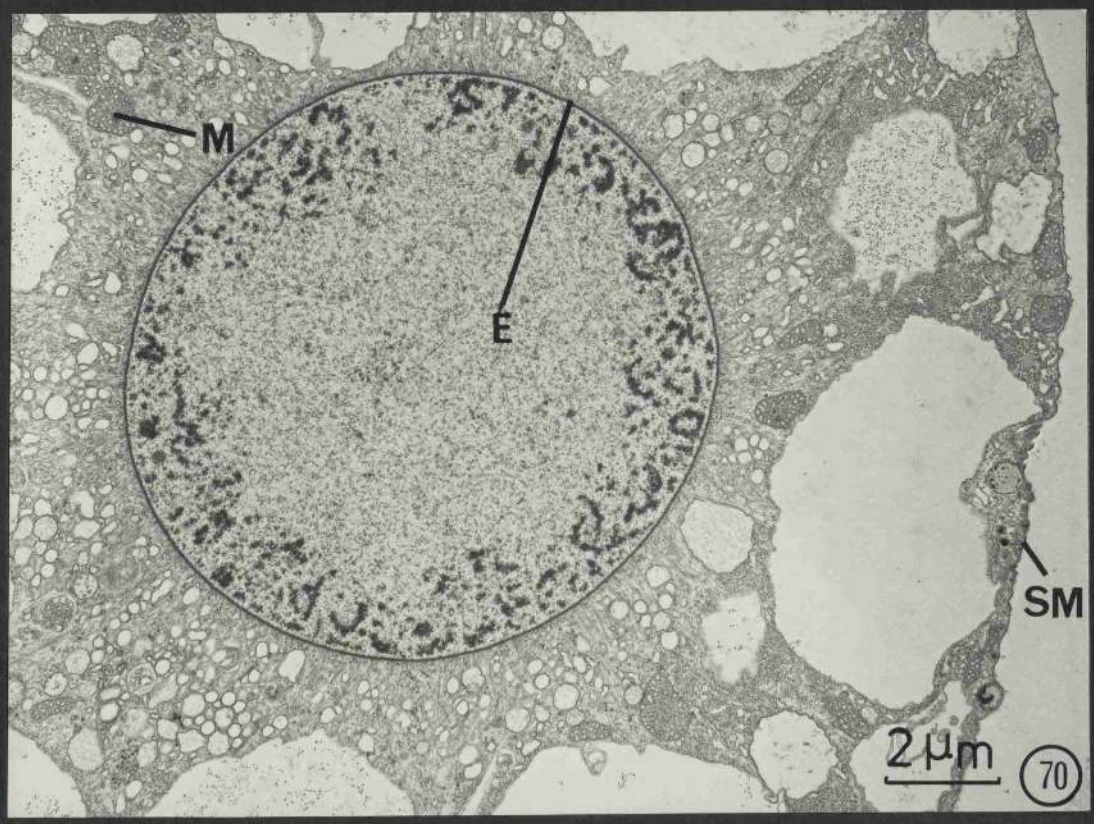
This section perpendicular to the nuclear envelope (E) shows tubules attached to it at their bases. Profiles of tubules sectioned longitudinally appear to he longer than after only 1 min of recovery (Plate 65).
x 30,000

PLATE 70

Microtubules are distributed over the whole of the nuclear envelope (E). The surface membrane (SM) has a smooth outline — no axopodia are present. Mitochondria (M) are present in the ectoplasm and in the layer of endoplasm bordering on the ectoplasm.
x 7,218


V
1 µm
68


E
0·5µm
69


M
E
SM
2µm
70

Plates 71-73 are micrographs of sections taken from organisms treated at -3°C for 6 hr and allowed to recover at room temperature for 2 min prior to fixation with cold fixative (Chapter VI).

PLATE 71

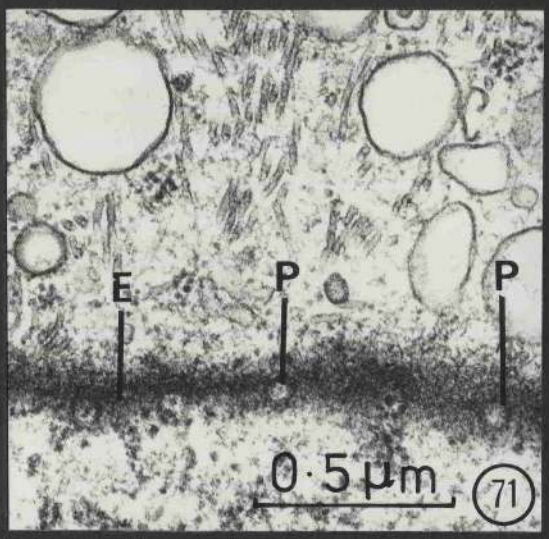
A "grazing" section through the nuclear envelope (E) reveals nuclear pores (P) in near-transverse section. The central region of these pores appears electron-lucent.
x 52,727

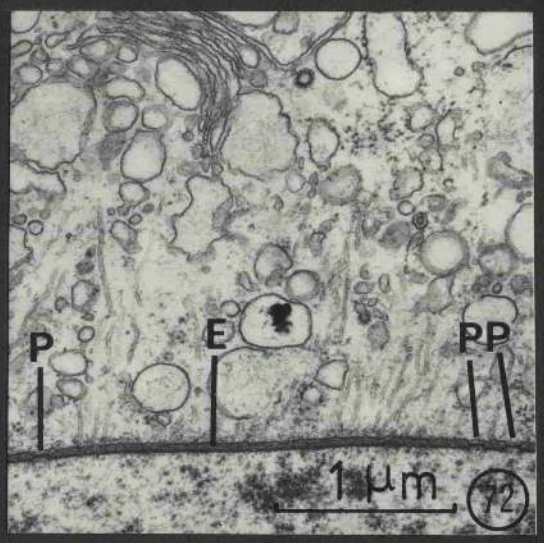
PLATE 72

A section perpendicular to the nuclear envelope (E) contains three nuclear pores (P). These pores contain less electron-dense material than the nuclear pores of organisms which have not been subjected to cold treatment (Plate 3). Microtubules appear to be attached at their bases to the nuclear envelope.
x 24,666

PLATE 73

This survey micrograph shows tubule attachment sites distributed over the nuclear envelope in a similar manner to their distribution over the surface of nuclear envelopes in organisms fixed with warmer fixative after the same period of recovery from cold treatment (Plate 70).
x 7,836


E
P
P
0·5µm
71


P
E
PP
1µm
72


2µm
73

Plates 74-77 are micrographs of sections through organisms treated at -3°C for 6 hr and allowed to recover at room temperature for 5 min prior to fixation.

PLATE 74

This section through the endoplasm reveals groups of tubules in transverse section. Some of the microtubules are arranged in the usual axonemal pattern, others are not. Tubules not in axonemal patterns are frequently seen at long and short link spacings from each other.
x 37,333

PLATE 75

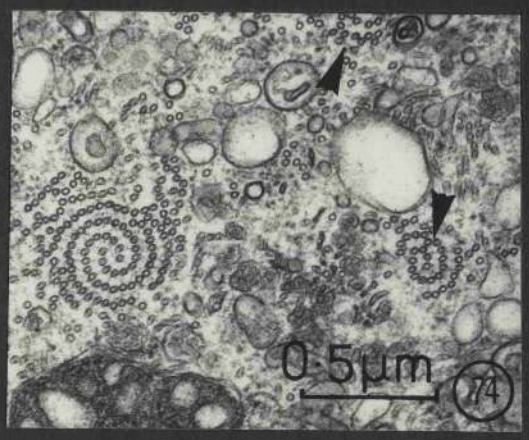
This highly magnified transverse section through a group of microtubules reveals the subfibres in the microtubule walls (arrows). One microtubule has apparently split open. Short links (L) are visible in a few instances.
x 211,764

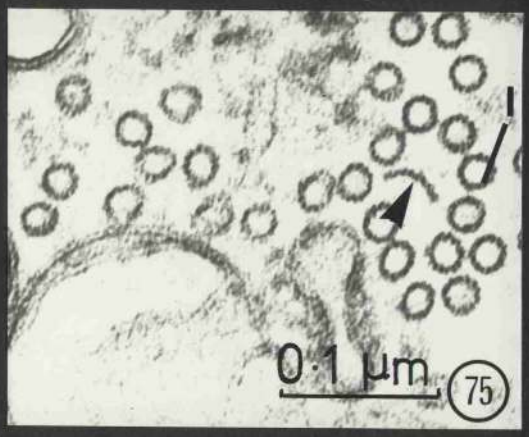
PLATE 76

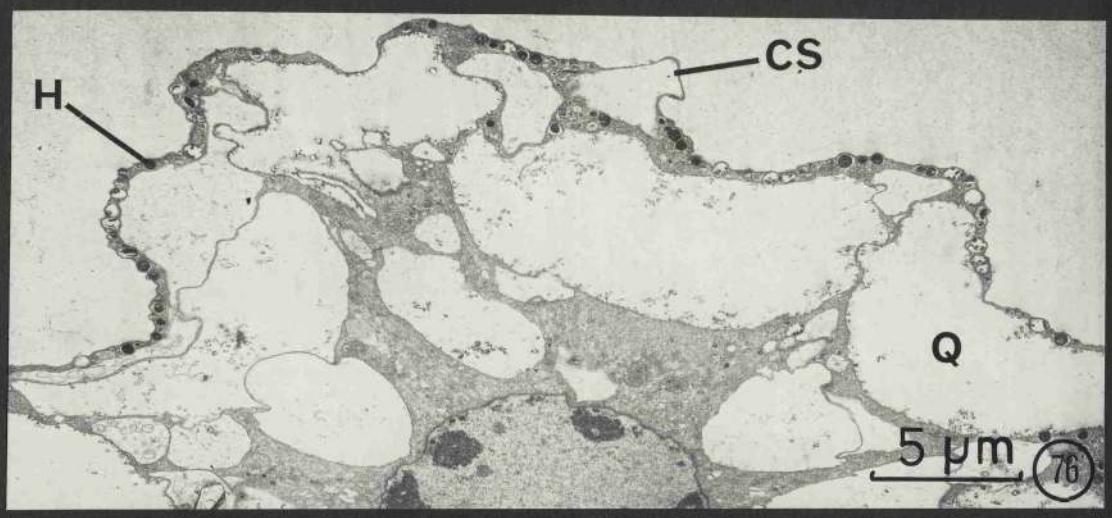
Axopodia do not appear to protrude from the cell surface (CS) of this organism. Haptocysts (H) are seen in the outer layer of ectoplasm. There are many ectoplasmic vacuoles (Q).
x 4,000

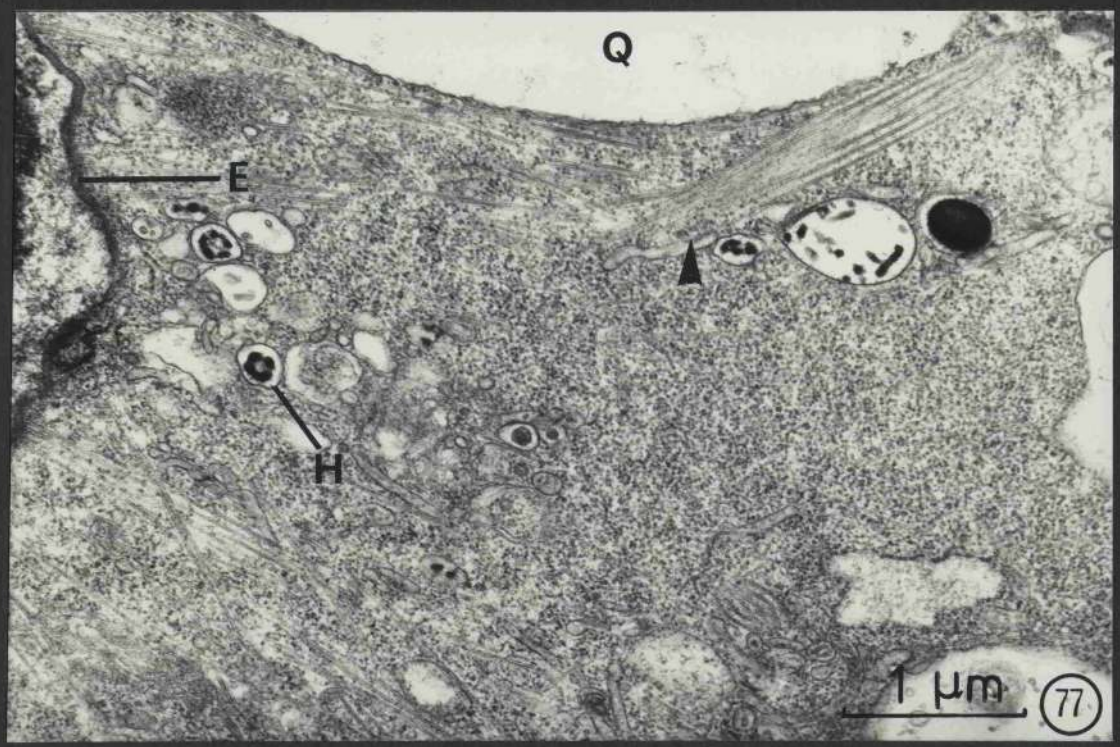
PLATE 77

A longitudinal section of an axoneme (arrow) which does not contact the nuclear envelope (E) at its base. The axoneme is not radially oriented, but lies tangentially to the membrane of a large ectoplasmic vacuole (Q). Haptocyst-like vesicles lie deep in the endoplasm.
x 24,722


0.5 µm
74


I
0.1 µm
75


CS
H
Q
5 µm
76


Q
E
H
1 µm
77

Plates 78-82 are micrographs of sections taken from organisms treated at -3°C for 6 hr and allowed to recover for 10 min prior to fixation.

PLATE 78

A transverse section of an axoneme with aberrant tubule packing.
x 22,307

PLATE 79

A transverse section of an axoneme with aberrant tubule packing.
x 43,200

PLATE 80

This survey micrograph shows the bases of many axonemes (arrows) which contact the surface of the nuclear envelope.
x 3,111

PLATE 81

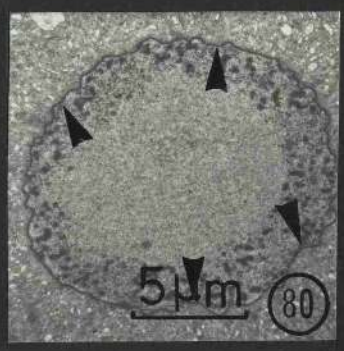
The base of this axoneme, sectioned longitudinally, contacts the nuclear envelope in the same way as the base of an axoneme from an untreated organism (Plate 2). Axonemes such as this are frequently radially oriented. Microtubule attachment points to the nuclear envelope are not distributed over most of the envelope as they were at earlier stages of recovery from cold treatment (Plates 67 and 70). Clusters of axonemal vesicles (V) occur near developing axonemes.
x 17,263

PLATE 82

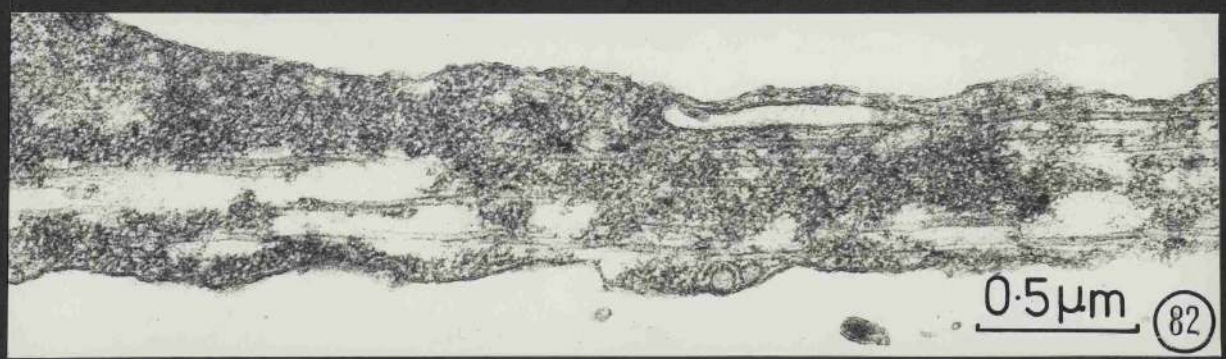
Axopodia protrude from the surface of the cell body. This longitudinal section of an axopodium reveals a sparse population of microtubules which are evidently not packed in the normal axonemal configuration.
x 43,157


1 µm
78


0·5µm
79


5µm
80


V
1 µm
81


0·5µm
82

Plates 83-87 are micrographs of sections taken from organisms treated at -3°C for 6 hr and allowed to recover at room temperature for 45 min prior to fixation.

PLATE 83

A transverse section through an axopodium shows several irregularly packed microtubules.
x 54,545

PLATE 84

A longitudinal section of the tip of a growing axopodium which contains many longitudinally oriented microtubules, haptocysts (H) and mitochondria (M).
x 33,333

PLATE 85

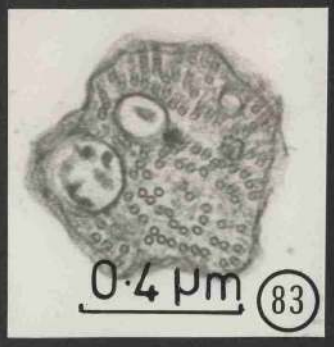
Shows a transverse section of an axoneme within the cell body. Adjacent to the axoneme lies a group of axonemal vesicles (V).
x 46,666

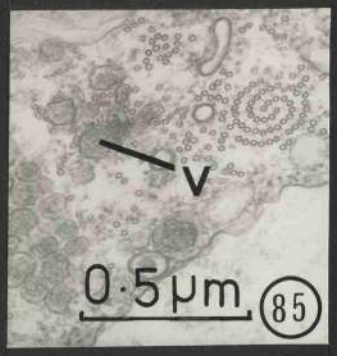
PLATE 86

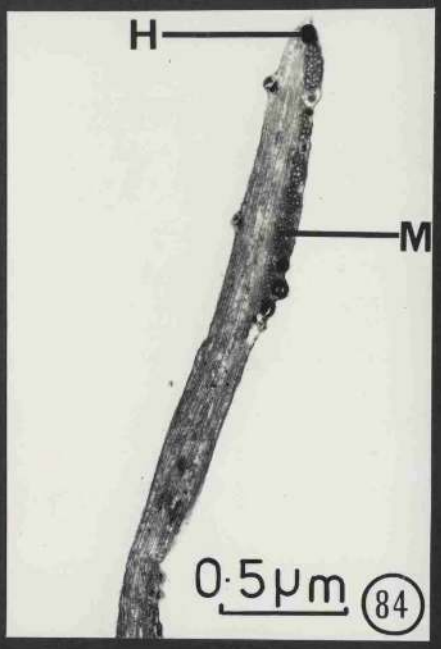
After 45 min of recovery small groupings of tubules are still not perfectly radially oriented (arrows) in this section through the endoplasm. This suggests that the reordering of all microtubules is not complete at this stage of recovery from cold.
x 26,666

PLATE 87

This longitudinal section through the base of a large axoneme (A) where it contacts the nuclear envelope reveals other tubules nearby which are not grouped into large axonemes. The appearance of these tubules also suggests that the ordering of all tubules is not complete by this stage.
x 26,666


0·4 µm
83


V
0·5 µm
85


H
M
0·5 µm
84


0·5 µm
86

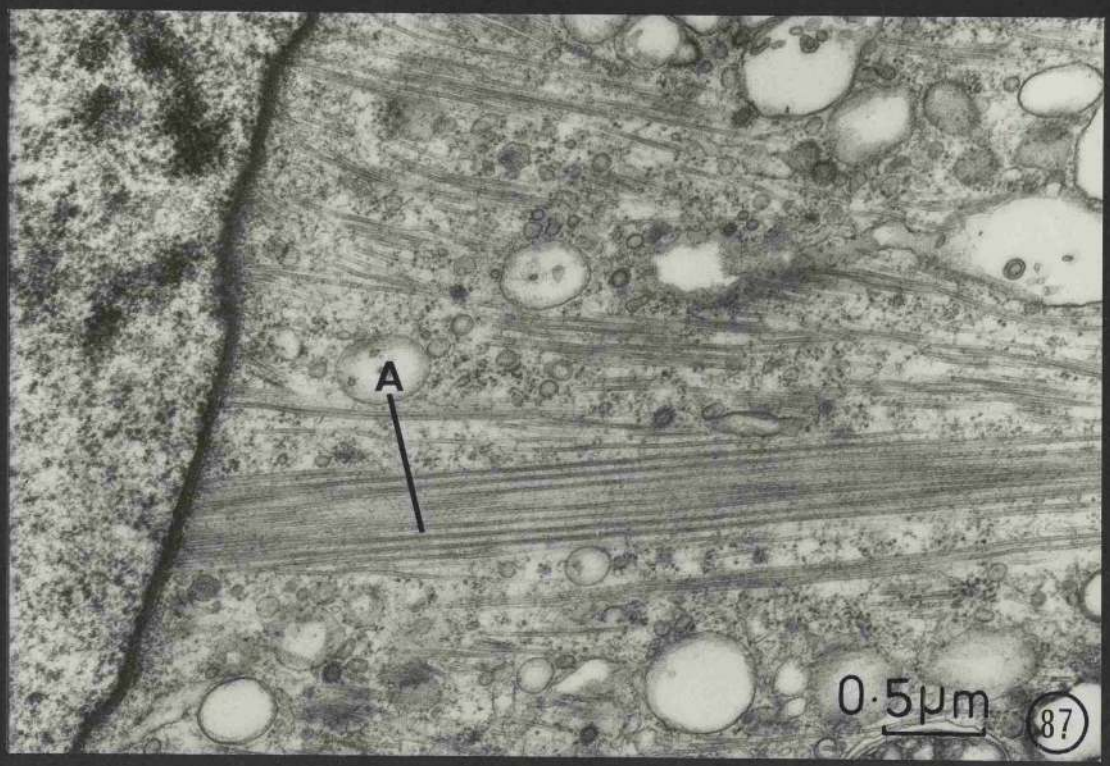

A
0·5 µm
87

PLATE 88

A living organism with axopodia retracted as a result of colchicine treatment.
x 483 (Nom)

PLATE 89

After washing several times in fresh culture medium the axopodia of the same organism as shown in Plate 88 have grown out again.
x 483 (Nom)

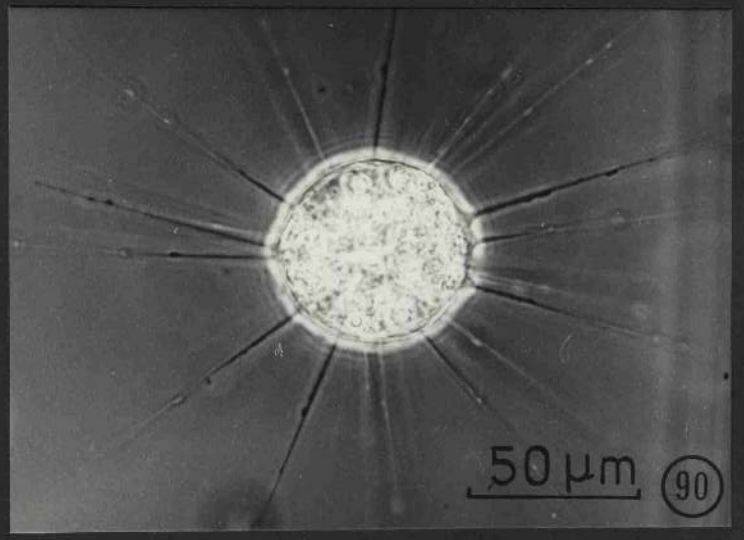
PLATE 90

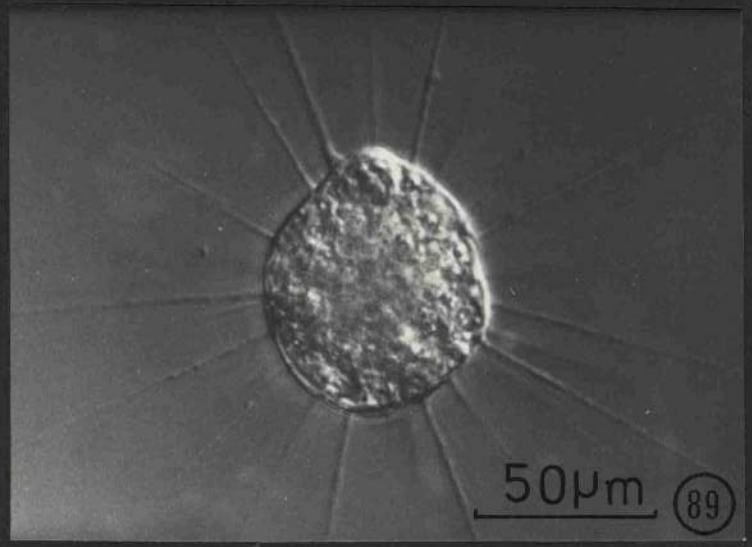
A living organism prior to treatment with E.G.T.A.
x 454 (P)

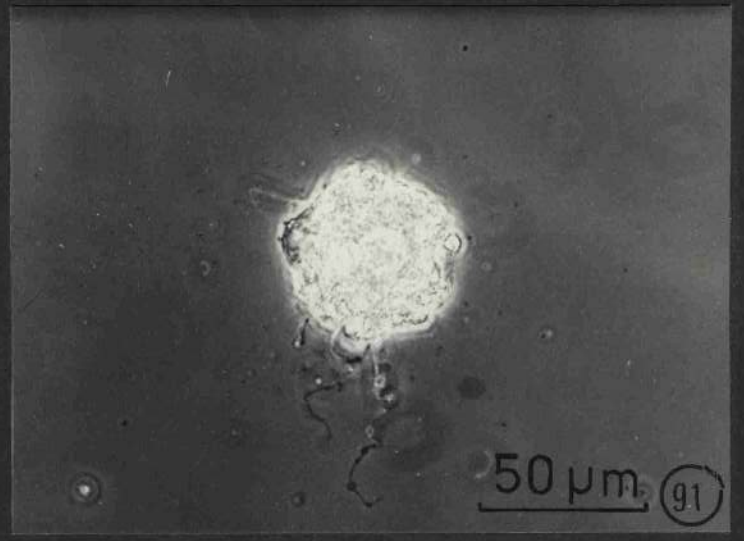
PLATE 91

Four minutes after treatment with a solution of E.G.T.A. a few damaged axopodia still remain.
x 454 (P)


50µm
88


50µm
90


50µm
89


50µm
91

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It is a great pleasure to thank all those who have helped me complete this thesis. Ideas, tuition in techniques, access to equipment, encouragement and constructive criticism have been given by many, quite freely, and in the most friendly manner.

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I wish to thank Professor M.S. Laverack for use of the Scanning Electron Microscope in the Gatty Marine Laboratory, Dr J. Starling and Professor Sir John Randall for their hospitality whilst I was working in the Zoology Department at Edinburgh University, and Dr K. Turvey of the St Andrews University Physics Department for his patient help with the determination of Young's Modulus for the axopodium.

C. D. O.